

PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁷ : A61K		A2	(11) International Publication Number: WO 00/67700
			(43) International Publication Date: 16 November 2000 (16.11.00)
(21) International Application Number: PCT/US00/12890			(74) Agents: POSORSKE, Laurence, H. et al.; Baker Botts, L.L.P., The Warner, 1299 Pennsylvania Avenue, N.W., Washington, DC 20004 (US).
(22) International Filing Date: 12 May 2000 (12.05.00)			
(30) Priority Data:			
60/133,866 12 May 1999 (12.05.99) US			
60/133,873 12 May 1999 (12.05.99) US			
60/133,869 12 May 1999 (12.05.99) US			(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).
60/133,865 12 May 1999 (12.05.99) US			
60/133,868 12 May 1999 (12.05.99) US			
60/133,867 12 May 1999 (12.05.99) US			
60/146,192 29 July 1999 (29.07.99) US			
(71) Applicant (for all designated States except US): UNITED STATES ARMY MEDICAL RESEARCH & MATERIEL CMD [US/US]; 504 Scott Street, Fort Detrick, MD 21702-5012 (US).			
(72) Inventors; and			
(75) Inventors/Applicants (for US only): SMITH, Leonard, A. [US/US]; Clarksburg, MD (US). BYRNE, Michael, P. [US/US]; Frederick, MD (US). MIDDLEBROOK, John, L. [US/US]; Middletown, MD (US). LAPENOTIERE, Hugh [US/US]; Charlestown, WV (US).			
(54) Title: RECOMBINANT VACCINE AGAINST BOTULINUM NEUROTOXIN			
(57) Abstract			
<p>This invention is directed to preparation and expression of synthetic genes encoding polypeptides containing protective epitopes of botulinum neurotoxin (BoNT). The invention is also directed to production of immunogenic peptides encoded by the synthetic genes, as well as recovery and purification of the immunogenic peptides from recombinant organisms. The invention is also directed to methods of vaccination against botulism using the expressed peptides.</p>			

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

Recombinant Vaccine Against Botulinum Neurotoxin

This application is based on U.S. Provisional Application Nos. 60/133,866, 60/133,868, 60/133,869, 60/133,865, 60/133,873, 60/133,867, all filed May 12, 1999, and U.S. Provisional Application No. 60/146,192, filed July 29, 1999, all of which are incorporated herein in their entirety.

BACKGROUND OF THE INVENTION

Field of the Invention

10 This invention is directed to preparation and expression of synthetic genes encoding polypeptides containing protective epitopes of botulinum neurotoxin (BoNT). The invention is also directed to methods of vaccination against botulism using the expressed peptides.

Related Art

15 The sporulating, obligate anaerobic, gram-positive bacillus *Clostridium* produces eight forms of antigenically distinct exotoxins. Tetanus neurotoxin (TeNT) is produced by *Clostridium tetani* while *Clostridium botulinum* produces seven different neurotoxins which are differentiated serologically by specific neutralization. The botulinum neurotoxins (BoNT) have been designated as
20 serotypes A, B, C₁, D, E, F, and G. Botulinum neurotoxins (BoNT) are the most toxic substances known and are the causative agents of the disease botulism. BoNT exert their action by inhibiting the release of the neurotransmitter acetylcholine at the neuromuscular junction (Habermann, E., et al., (1986), "Clostridial Neurotoxins: Handling and Action at the Cellular and Molecular Level," *Cur. Top. Microbiol. Immunol.*, **129**:93-179; Schiavo, G., et al., (1992a), "Tetanus and Botulinum-B Neurotoxins Block Neurotransmitter Release by Proteolytic Cleavage of Synaptobrevin," *Nature*, **359**:832-835; Simpson, L.L., (1986), "Molecular Pharmacology of Botulinum Toxin and Tetanus Toxin," *Annu. Rev. Pharmacol. Toxicol.*, **26**:427-453) which leads to a state of flaccid paralysis. Indeed, only a few
25 molecules of toxin can abolish the action of a nerve cell. Polyclonal antibodies derived for a specific neurotoxin can neutralize the toxic effects of that toxin but will not cross-neutralize another toxin serotype. Thus, to protect against all seven toxins, one needs seven vaccines.
30

Botulinum neurotoxins are translated as a single 150 kDa polypeptide chain and then posttranslationally nicked, forming a dichain consisting of a 100 kDa heavy chain and a 50 kDa light chain which remain linked by a disulfide bond (DasGupta, B.R., et al., (1972), "A Common Subunit Structure in *Clostridium botulinum* Type A, B, and E Toxins," *Biophys. Res. Commun.*, **48**:108-112; DasGupta, B.R., (1989), "The Structure of Botulinum Neurotoxins," *Botulinum Neurotoxin and Tetanus Toxin*, (Simpson, L.L., Ed.), pp. 53-67, Academic Press, New York). Most of the clostridial strains contain specific endogenous proteases which activate the toxins at a protease-sensitive loop located approximately one third of the way into the molecule from the amino-terminal end. Upon reduction and fractionation (electrophoretically or chromatographically), the two chains can be separated; one chain has a Mr of ~100 kDa and is referred to as the heavy chain while the other has a Mr ~50 kDa and is termed the light chain.

The mechanism of nerve intoxication is accomplished through the interplay of three key events, each of which is performed by a separate portion of the neurotoxin protein. First, the carboxy half of the heavy chain (fragment C or H_C is required for receptor specific binding to cholinergic nerve cells (Black, J.D., et al., (1986), "Interaction of ¹²⁵I-botulinum Neurotoxins with Nerve Terminals. I. Ultrastructural Autoradiographic Localization and Quantitation of Distinct Membrane Acceptors for Types A and B on Motor Nerves," *J. Cell Biol.*, **103**:521-534; Nishiki, T.-I., et al., (1994), "Identification of Protein Receptor for *Clostridium botulinum* Type B Neurotoxin in Rat Brain Synaptosomes," *J. Biol. Chem.*, **269**:10498-10503; Shone, C.C., et al., (1985), "Inactivation of *Clostridium botulinum* Type A Neurotoxin by Trypsin and Purification of Two Tryptic Fragments. Proteolytic Action Near the COOH-terminus of the Heavy Subunit Destroys Toxin-Binding Activity," *Eur. J. Biochem.*, **151**:75-82). There is evidence suggesting that polysialogangliosides (van Heyningen, W.E., (1968), "Tetanus," *Sci. Am.*, **218**:69-77) could act as receptors for the toxins but the data supporting a specific receptor remains equivocal (Middlebrook, J.L., (1989), "Cell Surface Receptors for Protein Toxins," *Botulinum Neurotoxins and Tetanus Toxin*, (Simpson, L.L., Ed.) pp. 95-119, Academic Press, New York). After binding, the toxin is internalized into an endosome through receptor-mediated endocytosis

(Shone, C.C., et al., (1987), "A 50-kDa Fragment from the NH₂-terminus of the Heavy Subunit of *Clostridium botulinum* Type A Neurotoxin Forms Channels in Lipid Vesicles, *Euro. J. Biochem.*, **167**:175-180). The amino terminal half of the heavy chain is believed to participate in the translocation mechanism of the light chain across the endosomal membrane (Simpson, 1986; Poulain, B., et al., (1991), "Heterologous Combinations of Heavy and Light Chains from Botulinum Neurotoxin A and Tetanus Toxin Inhibit Neurotransmitter Release in *Aplysia*," *J. Biol. Chem.*, **266**:9580-9585; Montal, M.S., et al., (1992), "Identification of an Ion Channel-Forming Motif in the Primary Structure of Tetanus and Botulinum Neurotoxins," *FEBS*, **313**:12-18). The low pH environment of the endosome may trigger a conformational change in the translocation domain, thus forming a channel for the light chain. The final event of intoxication involves enzymatic activity of the light chain, a zinc-dependent endoprotease (Schiavo, 1992a; Schiavo, G., et al., (1992b), "Tetanus Toxin is a Zinc Protein and its Inhibition of Neurotransmitter Release and Protease Activity Depend on Zinc," *EMBO J.*, **11**:3577-3583), on key synaptic vesicle proteins (Schiavo, 1992a; Oguma, K., et al., (1995), "Structure and Function of *Clostridium botulinum* Toxins," *Microbiol. Immunol.*, **39**:161-168; Schiavo, G., et al., (1993), "Identification of the Nerve Terminal Targets of Botulinum Neurotoxin Serotypes A, D, and E," *J. Biol. Chem.*, **268**:23784-23787; Shone, C.C., et al., (1993), "Proteolytic Cleavage of Synthetic Fragments of Vesicle-Associated Membrane Protein, Isoform-2 by Botulinum Type B Neurotoxin," *Eur. J. Biochem.*, **217**:965-971) necessary for neurotransmitter release. The light chains of BoNT serotypes A, C₁, and E cleave SNAP-25 (synaptosomal-associated protein of M25,000), serotypes B, D, F, and G cleave VAMP/synaptobrevin (synaptic vesicle-associated membrane protein); and serotype C₁ cleaves syntaxin. Inactivation of SNAP-25, VAMP, or syntaxin by BoNT leads to an inability of the nerve cells to release acetylcholine resulting in neuromuscular paralysis and possible death, if the condition remains untreated.

Human botulism poisoning is generally caused by type A, B, E or rarely, by type F toxin. Type A and B are highly poisonous proteins which resist digestion by the enzymes of the gastrointestinal tract. Foodborne botulism poisoning is caused by the toxins present in contaminated food, but wound and infant botulism are caused

by *in vivo* growth in closed wounds and the gastrointestinal tract respectively. The toxins primarily act by inhibiting the neurotransmitter acetylcholine at the neuromuscular junction, causing paralysis. Another means for botulism poisoning to occur is the deliberate introduction of the toxin(s) into the environment as might
5 occur in biological warfare. When the cause of botulism is produced by toxin rather than by *in vivo* infection the onset of neurologic symptoms is usually abrupt and occurs within 18 to 36 hours after ingestion. The most common immediate cause of death in respiratory failure due to diaphragmatic paralysis. Home canned foods are the most common sources of toxins. The most frequently implicated toxin is toxin
10 A, which is responsible for more than 50% of morbidity resulting from botulinum toxin.

Because even small amounts of botulinal toxin can cause serious illness, persons such as laboratory workers who are exposed to toxin must learn to handle all samples that may contain toxin with extreme care. It is also suggested that such
15 workers be protected from illness by vaccination against the toxins. Furthermore, persons exposed to conditions in which botulism toxins might be in the environment which might be inhaled or ingested, such as military personnel, need to be protected from the toxin.

Agents that abolish the action of BoNT have been investigated since the
20 1940s. Early work at Fort Detrick in the 1940s lead to the development of a toxoid vaccine to protect against serotypes A, B, C₁, D, and E toxins. The toxoid vaccine was manufactured by growing five *Clostridium botulinum* strains, extracting and precipitating the toxin from the growth media after cell lysis. Formalin was added to the crude preparation to inactivate the neurotoxin. Residual formalin was left in the
25 vaccine product to ensure the toxin remains non-toxic. The product was adsorbed to aluminum hydroxide and blended. Currently, a pentavalent toxoid vaccine against serotypes A through E (Anderson, J.H., et al., (1981), "Clinical Evaluation of Botulinum Toxoids," *Biomedical Aspects of Botulism*, (Lewis, G.E., Ed.), pp. 233-246, Academic Press, New York; Ellis, R. J., (1982), "Immunobiologic Agents and
30 Drugs Available from the Centers for Disease Control. Descriptions, Recommendations, Adverse Reactions and Serologic Response," 3rd ed., Centers for Disease Control. Atlanta, GA; Fiock, M.A., et al., (1963), "Studies of Immunities to

Toxins of *Clostridium Botulinum*. IX. Immunologic Response of Man to Purified Pentavalent ABCDE Botulinum Toxoid," *J. Immunol.*, **90**:697-702; Siegel, L.S., (1988), "Human Immune Response to Botulinum Pentavalent (ABCDE) Toxoid Determined by a Neutralization Test and by an Enzyme-Linked Immunosorbent Assay," *J. Clin. Microbiol.*, **26**:2351-2356), available under Investigational New Drug (IND) status, is used to immunize specific populations of at-risk individuals, i.e., scientists and health care providers who handle BoNT and our armed forces who may be subjected to weaponized forms of the toxin. Though serotypes A, B, and E are most associated with botulism outbreaks in humans, type F has also been diagnosed (Midura, T.F., et al., (1972), "*Clostridium botulinum* Type F: Isolation from Venison Jerky," *Appl. Microbiol.*, **24**:165-167; Green, J., et al., (1983), "Human Botulism (Type F) – A Rare Type," *Am. J. Med.*, **75**:893-895; Sonnabend, W.F., et al., (1987), "Intestinal Toxicoinfection by *Clostridium botulinum* Type F in an Adult. Case Associated with Guillian-Barre Syndrome," *Lancet*, **1**:357-361; Hatheway, C.L., (1976), "Toxoid of *Clostridium botulinum* Type F: Purification and Immunogenicity Studies," *Appl. Environ. Microbiol.*, **31**:234-242). A separate monovalent toxoid vaccine against BoNTF is available under IND status. Hatheway demonstrated that the BoNTF toxoid could protect guinea pigs against a homologous challenge (Wadsworth, J.D.F., et al., (1990), "Botulinum Type F Neurotoxin," *Biochem. J.*, **268**:123-128).

Even though toxoid vaccines are available, there are numerous shortcomings with their current use and ease of production. First, because *C. botulinum* is a spore-former, a dedicated facility is required to manufacture a toxin-based product. The requirement for a dedicated manufacturing facility is not trivial. It is extremely costly to renovate and upgrade an existing facility or to build a new one and then to maintain the facility in accordance with current Good Manufacturing Practices (cGMP) to manufacture one vaccine. Second, the yields of toxin production from *C. botulinum* are relatively low. Third, the toxoiding process involves handling large quantities of toxin and thus is dangerous, and the added safety precautions increase the cost of manufacturing. Fourth, the toxoid product for types A-E consists of a crude extract of clostridial proteins that may influence immunogenicity or reactivity of the vaccine, and the type F toxoid is only partially purified (IND 5077). Fifth,

because the toxoiding process involves the use of formaldehyde, which inactivates the toxin, and residual levels of formaldehyde (not to exceed 0.02%) are part of the product formulation to prevent reactivation of the toxin, the vaccine is reactogenic. An additional component of the toxoid vaccines is the preservative thimerosal
5 (0.01%), which also increases the reactogenicity of the product.

The development of a new-generation, recombinant vaccine could alleviate many of the problems associated with the toxoid. A recombinant vaccine would eliminate the need for a dedicated manufacturing facility. Presently, many cGMP facilities are in existence and available that could manufacture a recombinant
10 product. There would be no need to culture large quantities of a hazardous toxin-producing bacterium. Production yields from a genetically engineered product is expected to be high. There would be no need to treat the vaccine with formalin because the product would be non-toxic from the outset. Recombinant products would be purer, less reactogenic, and more fully characterized. Thus, the cost of a
15 recombinant product would be expected to be much lower than a toxoid because there would be no expenditures required to support a dedicated facility, and the higher production yields would reduce the cost of the vaccine product.

SUMMARY OF THE INVENTION

20 It is an object of this invention to provide immunogenic peptides capable of eliciting protective immunity against botulinum neurotoxin of serotypes A-G.

It is another object of this invention to provide vaccines capable of eliciting protective immunity against botulinum neurotoxin, where the vaccines do not act as neurotoxins themselves.

25 It is yet another object of this invention to provide methods for preparing non-toxic peptides for use in vaccines against botulinum neurotoxin by growing recombinant organisms which express the peptides.

It is still another object of this invention to provide methods for fast and efficient purification of the non-toxic peptides from cultures of recombinant
30 organisms.

These and other objects are met by one or more of the following embodiments of the present invention

In one embodiment, this invention provides a nucleic acid encoding the carboxy-terminal portion of the heavy chain (HC) of botulinum neurotoxin (BoNT), the BoNT being selected from the group consisting of BoNT serotype A, BoNT serotype B, BoNT serotype C1, BoNT serotype D, BoNT serotype E, BoNT serotype F, and BoNT serotype G, wherein said nucleic acid is expressable in a recombinant organism selected from *Escherichia coli* and *Pichia pastoris*. Preferably, the nucleic acid comprises a nucleic acid sequence selected from SEQ ID No:1 (serotype A), SEQ ID No:7 (serotype B), SEQ ID No:9 (serotype C1), SEQ ID No:11 (serotype D), SEQ ID No:13 (serotype E), SEQ ID No:15 (serotype F), and SEQ ID No:17 (serotype G). In an alternative preferred embodiment, the nucleic acid encodes an HC amino acid sequence of BoNT selected from SEQ ID No:2 (serotype A), SEQ ID No:8 (serotype B), SEQ ID No:10 (serotype C1), SEQ ID No:12 (serotype D), SEQ ID No:14 (serotype E), SEQ ID No:16 (serotype F), and SEQ ID No:18 (serotype G).

In another embodiment, this invention provides a nucleic acid encoding the amino-terminal portion of the heavy chain (HN) of botulinum neurotoxin (BoNT), the BoNT being selected from the group consisting of BoNT serotype B, BoNT serotype C1, BoNT serotype D, BoNT serotype E, BoNT serotype F, and BoNT serotype G, wherein said nucleic acid is expressable in a recombinant organism selected from *Escherichia coli* and *Pichia pastoris*. In a preferred embodiment, the nucleic acid comprises a nucleic acid sequence selected from SEQ ID No:21 (serotype B), SEQ ID No:23 (serotype C1), SEQ ID No:25 (serotype D), SEQ ID No:27 (serotype E), SEQ ID No:29 (serotype F), and SEQ ID No:31 (serotype G). Alternatively, the nucleic acid encodes an HN amino acid sequence of BoNT selected from SEQ ID No:22 (serotype B), SEQ ID No:24 (serotype C1), SEQ ID No:26 (serotype D), SEQ ID No:28 (serotype E), SEQ ID No:30 (serotype F), and SEQ ID No:32 (serotype G).

Preferably, the nucleic acid of this invention is a synthetic nucleic acid. In a preferred embodiment, the sequence of the nucleic acid is designed by selecting at least a portion of the codons encoding HC from codons preferred for expression in a host organism, which may be selected from gram negative bacteria, yeast, and mammalian cell lines; preferably, the host organism is *Escherichia coli* or *Pichia*

pastoris. In another preferred embodiment, the nucleic acid sequence encoding HC is designed by selecting codons encoding HC which codons provide HC sequence enriched in guanosine and cytosine residues. More preferably, nucleic acid encoding HC or HN is expressed in a recombinant host organism with higher yield
5 than a second nucleic acid fragment encoding the same HC sequence, said second nucleic acid fragment having the wild-type *Clostridium botulinum* sequence of HC.

In yet another embodiment, this invention provides an expression vector comprising the nucleic acid of this invention, whereby HC and/or HN is expressed upon transfection of a host organism with the expression vector. Another
10 embodiment of this invention provides a method of preparing a polypeptide comprising the carboxy-terminal portion of the heavy chain (HC) of botulinum neurotoxin (BoNT) or the amino-terminal portion of the heavy chain (HN) of botulinum neurotoxin (BoNT) selected from the group consisting of BoNT serotype A, BoNT serotype B, BoNT serotype C, BoNT serotype D, BoNT serotype E, BoNT
15 serotype F, and BoNT serotype G, said method comprising culturing a recombinant host organism transfected with the expression vector of of this invention under conditions wherein HC or HN is expressed. Preferably, the recombinant host organism is a eukaryote. In another preferred embodiment, the method of this invention further comprises recovering insoluble protein from the host organism,
20 whereby a fraction enriched in HC or HN is obtained. Preferably, the host organism is *Pichia pastoris*.

In still another embodiment, this invention provides an immunogenic composition comprising the carboxy-terminal portion of the heavy chain (HC) of botulinum neurotoxin (BoNT) selected from the group consisting of BoNT serotype
25 A, BoNT serotype B, BoNT serotype C, BoNT serotype D, BoNT serotype E, BoNT serotype F, and BoNT serotype G. Preferably, the immunogenic composition is prepared by culturing a recombinant organism transfected with an expression vector encoding HC. More preferably, the immunogenic composition is prepared by a method wherein an insoluble protein fraction enriched in HC is recovered from said
30 recombinant organism.

In yet another embodiment, this invention provides an immunogenic composition comprising the amino-terminal portion of the heavy chain (HN) of

botulinum neurotoxin (BoNT) selected from the group consisting of BoNT serotype A, BoNT serotype B, BoNT serotype C, BoNT serotype D, BoNT serotype E, BoNT serotype F, and BoNT serotype G. Preferably, the immunogenic composition comprising HN is prepared by culturing a recombinant organism transfected with an expression vector encoding HN. More preferably, the immunogenic composition is prepared from an insoluble protein fraction enriched in HN which is recovered from the recombinant organism.

In still another embodiment, this invention provides an immunogenic composition comprising a polypeptide comprising epitopes contained in the carboxy-terminal portion of the heavy chain (HC) of botulinum neurotoxin (BoNT) and/or the amino-terminal portion of the heavy chain (HN) of botulinum neurotoxin (BoNT) selected from the group consisting of BoNT serotype A, BoNT serotype B, BoNT serotype C, BoNT serotype D, BoNT serotype E, BoNT serotype F, and/or BoNT serotype G, said epitopes eliciting protective immunity toward the respective BoNT serotype. Preferably, the immunogenic composition elicits an ELISA response to the respective BoNT serotype(s) in an animal which is detectable in serum from the animal even when the serum is diluted 100-fold.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the sequence for a synthetic gene encoding the H_C fragment of BoNT serotype A and the encoded amino acids sequence.

Figure 2 shows the sequence for a synthetic gene encoding the H_C fragment of BoNT serotype A and the encoded amino acids sequence.

Figure 3 shows the sequence for a synthetic gene encoding the H_C fragment of BoNT serotype A and the encoded amino acids sequence.

Figure 4 shows the sequence for a synthetic gene encoding the H_C fragment of BoNT serotype B and the encoded amino acids sequence.

Figure 5 shows the sequence for a synthetic gene encoding the H_C fragment of BoNT serotype C and the encoded amino acids sequence.

Figure 6 shows the sequence for a synthetic gene encoding the H_C fragment of BoNT serotype D and the encoded amino acids sequence.

Figure 7 shows the sequence for a synthetic gene encoding the H_C fragment of BoNT serotype E and the encoded amino acids sequence.

Figure 8 shows the sequence for a synthetic gene encoding the H_C fragment of BoNT serotype E and the encoded amino acids sequence.

5 Figure 9 shows the sequence for a synthetic gene encoding the H_C fragment of BoNT serotype F and the encoded amino acids sequence.

Figure 10 shows the sequence for a synthetic gene encoding the H_C fragment of BoNT serotype G and the encoded amino acids sequence.

10 Figure 11 shows the sequence for a synthetic gene encoding the H_N fragment of BoNT serotype A and the encoded amino acids sequence.

Figure 12 shows the sequence for a synthetic gene encoding the H_N fragment of BoNT serotype B and the encoded amino acids sequence.

Figure 13 shows the sequence for a synthetic gene encoding the H_N fragment of BoNT serotype C and the encoded amino acids sequence.

15 Figure 14 shows the sequence for a synthetic gene encoding the H_N fragment of BoNT serotype D and the encoded amino acids sequence.

Figure 15 shows the sequence for a synthetic gene encoding the H_N fragment of BoNT serotype E and the encoded amino acids sequence.

20 Figure 16 shows the sequence for a synthetic gene encoding the H_N fragment of BoNT serotype F and the encoded amino acids sequence.

Figure 17 shows the sequence for a synthetic gene encoding the H_N fragment of BoNT serotype G and the encoded amino acids sequence.

Figure 18 shows the sequence for a synthetic gene encoding the H_C fragment of BoNT serotype F and the encoded amino acids sequence.

25 Figure 19 shows (A) AT base content of a putative fragment C region in native *C. botulinum* DNA. (B) Reduction at AT content after the first design (rBoNTF(Hc)1) of the synthetic gene. (C) AT content of the final gene design (rBoNTF(Hc)2) used to express recombinant rBoNTF(Hc) in *P. pastoris*.

30 Figure 20 shows (A) SDS-PAGE and (B) Western blot of samples at various steps along the rBoNTF(Hc) purification. Lanes from both figures are identical except lane 1, where SDS-PAGE shows Novex mark 12 wide-range molecular weight markers and Western blot shows Novex See Blue prestained molecular

weight markers. Lane 2 is the cell lysate, lane 3 is the cell extract, lane 4 is the cell extract after dialysis, lane 5 is pool of rBoNTF(Hc) positive fractions after Mono S column chromatography, and lane 6 is pool of rBoNTF(Hc)-positive fractions after hydrophobic interaction chromatography.

5 Figure 21 shows purification of rBoNTF(Hc) by sequential chromatography. (A) Mono S cation exchange chromatography of extract from *P. pastoris*. Proteins were eluted with increasing NaCl gradient. Fractions positive for rBoNTF(Hc) by Western analysis were pooled individually and subjected to hydrophobic interaction chromatography (B) and proteins were eluted with a decreasing ammonium sulfate
10 gradient. In both panels, protein monitored by A280nm is recorded on the left axis and elution conditions are recorded on the right axis, with the gradient trace laid over the chromatogram.

 Figure 22 shows CD spectra of purified soluble (—) and resolubilized (--) rBoNTF(Hc) at 30 μ g/ml (0.62 μ M) in 10 mM sodium phosphate, pH 7.0 in a 1-cm
15 path length cell. Spectra were the average of four accumulations, scanned from 260 to 200 nm at a scan rate of 10 nm/min with a 2-s response and a 1-nm bandwidth. The temperature was maintained at 20°C using a Peltier thermocontrol device.

20 DETAILED DESCRIPTION OF THE EMBODIMENTS OF THIS INVENTION

 The present inventors have determined that animals, including primates, may be protected from the effects of botulinum neurotoxin (BoNT) by immunization with fragments of the botulinum neurotoxin protein expressed by recombinant organisms. Specifically, peptides comprising protective epitopes from the receptor
25 binding domain and/or the translocation domain, found in the carboxy terminal and the amino terminal portions of the heavy chain of the BoNT protein, respectively, are expressed by recombinant organisms transfected with expression vectors encoding the peptides for each serotype of BoNT. Immunization with these recombinantly produced peptides will elicit antibodies capable of protecting animals
30 against intoxication with the BoNT of the respective serotype.

 This invention provides a genetically engineered vaccine for protection against botulism. The vaccine comprises fragments of the A and B toxins known as

"C fragments" (H_C domain). It is now possible to produce the H_C fragments of the A and B toxins in *E. coli* using gene segment constructs of the HC fragment or an HC polypeptide fused to *E. coli* maltose binding protein. It has been found that the fusion product provides excellent protection against the native toxin challenge. The invention provides plasmids and recombinant proteins for use as vaccines to provide protection against toxins of *Clostridium botulinum*.

Kozaki et al. in "Antibodies against Botulism Neurotoxin", L.L. Simpson, ed., 1989, Academic Press, New York, suggested that a protective epitope might be present in the 50 kDa carboxyl terminus (HC) region of the protein. Thompson et al. (1990, *Eur. J. Biochem.* **189**:73-81) deduced the amino acid sequence for the serotype A botulinum toxin. DasGupta, et al. (1990, *Biochemie*, **72**:661-664), identified the "nick" site for post-translational cleavage of the expressed toxin polypeptide, from which the sequence of the heavy chain can be deduced as follows (see also Krieglstein, et al., 1994, *J. Protein Chem.*, **13**:49-57):

ALNDLCIKVNNWDLFFSPSEDNFTNDLNKGEEITSDTNIEAAEENISL
DLIQYYLTFNFDNEPENISIEENLSSDIIGQLELMPNIERFPNGKKYELDKYTM
FHYLRAQEFEGHKSRIALTNSVNEALLNPSRVYTFFSSDYVKKVNKATEAA
MFLGWVEQLVYDFTDETSEVSTTDKIADITHIIPYIGPALNIGMLYKDDFVGA
LIFSGAVILLEFIPEIAIPVLGTFALVSYIANKVLTQTVQTNALSKRNEKWDEV
YKYIVTNWLAKVNTQIDLRKKMKEALENQAEATKAIINYQYNQYTEEEKN
NINFNIDDLSSKLNESINKAMININKFLNQCSVSYLMNSMIPYGVKRLEDFDA
SLKDALLKYIRDNYGTLIGQVDRLKDKVNNTLSTDIPFQLSKYVDNQRLST
FTEYIKNIINTSILNLRYESNHLIDLSRYASKINIGSKVNFDPIDKNQIQLFNLES
SKIEVILKNAIVYNSMYENFSTSFWRIPKYFNSISLNNEYTIINCMENNSGWK
VSLNYGEIIWTLQDTQEIQRVVFYKYSQMINISDYINRWIFVTITNRLNNSKI
YTINGRLIDQKPISNLGNIHASNNIMFKLDGCRDTHRYIWIWIKYFNLFDKELNE
KEIKDLYDNQSNISGILKDFWGDYLYQYDKPYYMILLYDPNKYVDVNNVGIR
GYMYLKGPRGSVMTTNIYLNSSLYRGTKFIIKKASGNKDNIVRNNDRVYIN
VVVKNKEYRLATNASQAGVEKILSALEIPDVGNLSQVVVMKSKNDQGITNK
CKMNLQDNNGNDIGFIGFHQFNIAKLVASNWNROTERSSRTLGCSEWFI
PVDDGWGERPL

Whelan et al. (*Appl. Environ. Microbiol.* **58**:2345-2354, 1992) have deduced the amino acid sequence for the serotype B botulinum toxin. Schmidt, et al. (1985, *Arch. Biochem. Biophys.*, **238**:544-548), provided N-terminal sequence information for the heavy chain resulting from post-translational cleavage of the expressed toxin. polypeptide, and the sequence of the heavy chain can be deduced from this information as follows:

APGICIDVDNEDLFFIADKNSFSDDL SKNERIEYNTQSNYIENDFPINEL
 ILDTDLISKIELPSENTESLTDFNVDVPVYEKQPAIKKIFTDENTIFQYLYSQTF
 10 PLDIRDISLTSSFDDALLFSNKVYSFFSMDYIKTANKVVEAGLFAGWVKQIV
 NDFVIEANKSNTMDKIADISLIVPYIGLALNVGNETAKGNFENAFEIAGASIL
 LEFPELLIPVVGAFLLSYIDNKNKIIKTIDNALT KRNEKWSDMYGLIVAQW
 LSTVNTQFYTIKEGMYKALNYQAQALEEIIKYRYNIYSEKEKSNINIDFNDIN
 SKLNEGINQAIDNINNFIN GCSVSYLMKKMIPLAVEKLLDFDNTLKKNLLNYI
 15 DENKLYLIGSAEYEKSKVNKYLKTIMPFDL SIYTNDTILEMFNKYNSEILNNI
 ILNLRYKDNNLIDLSGYGAKVEVYDGV ELNDKNQFKLTSSANSKIRTTQNQ
 NIIFNSVFLDFSVSFWIRIPKYKNDGIQNYIHNEYTIINCMKNNSGWKISIRGRI
 IWTLDINGKTKSVFFEYNIREDISEYINRWFFVTITNNLNNAKIYINGKLESN
 TDIKDIREVIANGEIIFKLDGDIDRTQFIWMKYFSIFNTELSQSNI EERYKIQSY
 20 SEYLKDFWGNPLMYNKEYYMFNAGNKNSYTIK LKKDSPVGEILTRSKYNQ
 NSKYINYRDLYIGEKFIIRKKSNSQSINDDIVRKEDYIYLDFFNLNQEW RVYT
 YKYFKKEEELFLAPISDSDEFYNTIQIKEYDEQPTYSCQLL FKKDEESTDEIGL
 IGIHRFYESGIVFEEVKDVFCISDWYLEVKRKPYNLKLGCNWQFIPKDEGWT
 E

25 Analogous post-translational cleavage for all BoNT serotypes produces analogous heavy chain and light chain structures (see Krieglstein, et al., 1994, *J. Protein Chem.*, **13**:49-57, and references cited therein).

Synthetic Gene Construction

Preliminary experiments indicated that the DNA sequence found in *C. botulinum* encoding the relevant BoNT fragments are not well expressed in typical recombinant hosts. Therefore, synthetic gene construction was undertaken, based on the amino acid sequence of the respective fragments.

Synthetic gene construction is a technique used to optimize for expression in heterologous host systems. The base composition (i.e., percent A+T or percent G+C) as well as the specific codons in a gene sequence play a role in determining whether a gene from one organism will be optimally expressed in a different
5 organism. There is a reason why certain codons are used and why some are not. Organisms will use the codons in which corresponding tRNAs are present. If the organisms do not use certain codons, they most likely lack those specific tRNAs. As it turns out, codons found in clostridial DNA (i.e., genes found in the genus of bacterial called *Clostridium*) are very unique both in terms of base composition (i.e.,
10 very high A+T base composition) and in the use of codons not normally found in *E. coli* or yeast.

Table 1 is a chart depicting codon usage in *Pichia pastoris*. This table was generated by listing the codons found in a number of highly expressed genes in *P. pastoris*. The codon data was obtained by sequencing the genes and then listing
15 which codons were found in the genes.

From Table 1, it is clear that the amino acid residues can be encoded for by multiple codons. When constructing synthetic genes using *P. pastoris* codon usage, it is preferred to use only those codons that are found in the naturally occurring genes in *P. pastoris*, and it should be attempted to keep them in the same ratio found
20 in the genes of the natural organism. When the clostridial gene has an overall A+T richness of greater than 70% and A+T regions that have spikes of A+T of 95% or higher, they have to be lowered for expression in expression systems like yeast. (Preferably, the overall A+T richness is lowered below 60% and A+T in spikes is also lowered to 60% or below). It is of course necessary to balance keeping the
25 same codon ratio (e.g., for glycine GGG was not found, GGA was found 22% of the time, GGT was found 74% of the time, GGC was found 3% of the time) with reducing the high A+T content. In the construction of the genes, it is preferred to keep the A+T spikes about 55%.

Considering codon usage for a number of organisms including *E. coli*, it
30 turns out that a synthetic gene using *E. coli* codon usage also expresses fairly well in *P. pastoris*. Similarly, a synthetic gene using *P. pastoris* codon usage also appears to express very well in *E. coli*.

Synthetic genes for the H_C fragments of botulinum neurotoxin serotypes A-G are shown in Figures 1-10, along with the amino acid sequences encoded by the synthetic genes. Synthetic genes for the H_N fragments of botulinum neurotoxin serotypes A-G are shown in Figures 11-17, along with the amino acid sequences encoded by the synthetic genes. Synthetic genes having alternative gene sequences may be prepared by following the guidance provided herein concerning codon selection. The amino acid sequence encoded by such synthetic genes will preferably be the sequence of one of the BoNT serotype proteins, or a fragment thereof which contains protective epitopes. Suitable fragments include the H_C fragments of BoNT serotypes A, B C₁, D, E, F, and G, and the H_N fragments of BoNT serotypes A, B, C₁, D, E, F, and G. Such alternative gene sequences are within the contemplation of this invention.

Also within the contemplation of this invention are proteins containing protective epitopes from both the N-terminal and the C-terminal domains of the respective serotype BoNT proteins. Such proteins may be prepared by fusing a sequence encoding the translocation domain (HN) to the sequence of the HC region. This may be accomplished by removing the restriction enzyme site of the 3' end of the translocation domain gene as well as the termination codon, and also removing the initiation codon, restriction enzyme site and any other nucleotides on the 5' end of the gene that are not part of the botulinum toxin gene. Then a common restriction enzyme site not found in either synthetic gene may be inserted on the 3' end of the H_N gene and the 5' end of the H_C gene, and this common restriction site may be used to fuse the two genes together.

Recombinant Peptide Production

The nontoxin fragment is very safe, will not require formalin treatment, and has been shown to produce significant immunity against the fully toxic parent molecule. There are two major advantages of the invention over the presently employed vaccine. First, the recombinantly-produced botulinum neurotoxin (rBoNT) protein fragments are completely nontoxic and, is thus, very safe. The fermentation of the host cell harboring the rBoNT gene (e.g., *Escherichia coli* or *Pichia pastoris*) will not require the high biological containment facilities presently needed to ferment the spore-forming *Clostridium botulinum* required for the

production of the toxoid vaccine. Second, the synthetic gene can be placed in high expression systems and used to make much larger quantities of the fragment than toxin produced by the parent organism, *Clostridium botulinum*. Thus, there will be immense cost savings because it will be easier and safer to produce much larger quantities of the vaccine than is now possible.

Synthetic genes as described herein may be transfected into suitable host organisms to create recombinant production organisms, and cultures of these recombinant organisms can then be used to produce immunogenic peptide fragments capable of conferring protective immunity against BoNT of the respective serotypes. Exemplary techniques for transfection and production of BoNT fragments are shown in the Examples. Alternative techniques are well documented in the literature (See, e.g., Maniatis, Fritsch & Sambrook, "Molecular Cloning: A Laboratory Manual" (1982); "DNA Cloning: A Practical Approach," Volumes I and II (D.N. Glover, ed., 1985); "Oligonucleotide Synthesis" (M.J. Gait, ed., 1984); "Nucleic Acid Hybridization" (B.D. Hames & S.J. Higgins, eds., 1985); "Transcription and Translation" (B.D. Hames & S.J. Higgins, eds., 1984); "Animal Cell Culture" (R.I. Freshney, ed., 1986); "Immobilized Cells and Enzymes" (IRL Press, 1986); B. Perbal, "A Practical Guide to Molecular Cloning" (1984), and Sambrook, et al., "Molecular Cloning: a Laboratory Manual" (1989)). Such techniques are explained fully in the literature, and modification of these techniques within the scope of this invention is within the skill in the art.

The synthetic gene for BoNT serotype B fragment H_C (see Figure 4A) has been inserted into the yeast expression vector pHIL-D4, and integrated into the chromosome of *Pichia pastoris* strain GS115. The expressed product (see amino acid sequence in Figure 4B) had the expected molecular weight as shown by denaturing polyacrylamide gel electrophoresis (PAGE) and Western blot analysis using antibodies directed against botulinum neurotoxin serotype B. The expressed recombinant BoNTB (H_C) elicited high antibody titers as judged by the Enzyme Linked Immunosorbent Assay (ELISA) and, more importantly, these circulating serum titers protected mice, guinea pigs, and non-human primates from challenges with active toxin. Industrial scale manufacturing processes (fermentation and

purification) have been completed and a pilot lot has been produced in compliance with cGMP.

The synthetic gene for BoNT serotype C fragment H_C (see Figure 5A) has been inserted into the yeast expression vector pHIL-D4, and integrated into the chromosome of *Pichia pastoris* strain GS115. The expressed product (see amino acid sequence in Figure 5B) had the expected molecular weight as shown by denaturing polyacrylamide gel electrophoresis (PAGE) and Western blot analysis using antibodies directed against botulinum neurotoxin serotype C. The expressed recombinant BoNTC (H_C) elicited high antibody titers as judged by the Enzyme Linked Immunosorbent Assay (ELISA) and, more importantly, these circulating serum titers protected mice from challenges with active toxin.

The synthetic gene for BoNT serotype D fragment H_C (see Figure 6A) has been inserted into the yeast expression vector pHIL-D4, and integrated into the chromosome of *Pichia pastoris* strain GS115. The expressed product (see amino acid sequence in Figure 6B) had the expected molecular weight as shown by denaturing polyacrylamide gel electrophoresis (PAGE) and Western blot analysis using antibodies directed against botulinum neurotoxin serotype D. The expressed recombinant BoNTD (H_C) elicited high antibody titers as judged by the Enzyme Linked Immunosorbent Assay (ELISA) and, more importantly, these circulating serum titers protected mice from challenges with active toxin.

The synthetic gene for BoNT serotype E fragment H_C (see Figure 7A) has been inserted into the yeast expression vectors pHILD2, pHILD3, and pPIC9K (see Figures 7B). A modified form of the synthetic gene in which an internal EcoRI site was removed and the gene was enlarged (see Figure 8) was inserted into the yeast vector pHIL-D4, and integrated into the chromosome of *Pichia pastoris* strain GS115. The expressed product (see amino acid sequence in Figure 8) had the expected molecular weight as shown by denaturing polyacrylamide gel electrophoresis (PAGE) and Western blot analysis using antibodies directed against botulinum neurotoxin serotype E. The expressed recombinant BoNTE (H_C) elicited high antibody titers as judged by the Enzyme Linked Immunosorbent Assay (ELISA) and, more importantly, these circulating serum titers protected mice from challenges with active toxin.

The synthetic gene for BoNT serotype F fragment H_C (see Figure 9A) has been inserted into the yeast expression vector pHIL-D4, and integrated into the chromosome of *Pichia pastoris* strain GS115. The initial step in the development of the rBoNTF(H_C) vaccine candidate was to design a gene which could satisfactorily be expressed in a pichia host. A synthetic gene encoding rBoNTF(H_C) was constructed to lower the inherent AT richness of the native clostridial gene and to remove any potentially rare codons. Clostridial genes having an AT content in excess of 65% or having an average AT content but containing AT-rich tracts usually contain multiple terminators/polyadenylation signals, which can result in premature termination of transcripts when expression is attempted in yeast (Romanos, M.A., et al., (1995), "Expression of Cloned Genes in Yeast," *DNA Cloning 2: Expression Systems*," (Glover D., et al., Eds.), Oxford Univ. Press, London). The synthetic gene in this study required two successive rounds of alterations before the yeast could properly produce full-length antigen. The expressed product (see amino acid sequence in Figure 9B) had the expected molecular weight as shown by denaturing polyacrylamide gel electrophoresis (PAGE) and Western blot analysis using antibodies directed against botulinum neurotoxin serotype F.

A previous study (Hatheway, 1976) demonstrated that the serotype F toxoid antigen needed to be at least partially purified to be efficacious. The same observation was noted with the rBoNTF(H_C) antigen produced in pichia cells as the crude cell lysate did not protect mice against a BoNTF challenge. The putative receptor-binding domain of BoNTF was purified from yeast and shown to be efficacious in a mouse model. The expressed recombinant BoNTF (H_C) elicited high antibody titers as judged by the Enzyme Linked Immunosorbent Assay (ELISA) and, more importantly, these circulating serum titers protected mice from challenges with active toxin.

The synthetic gene for BoNT serotype G fragment H_C (see Figure 10A) has been inserted into the yeast expression vector pHIL-D4, and integrated into the chromosome of *Pichia pastoris* strain GS115. The expressed product (see amino acid sequence in Figure 10B) had the expected molecular weight as shown by denaturing polyacrylamide gel electrophoresis (PAGE) and Western blot analysis

using antibodies directed against botulinum neurotoxin serotype G. The expressed recombinant BoNTG (H_C) elicited high antibody titers as judged by the Enzyme Linked Immunosorbent Assay (ELISA) and, more importantly, these circulating serum titers protected mice from challenges with active toxin.

5 When purifying a protein for the first time, it is important to generate a viable means for identifying which fractions contain product. If the protein of interest is not an enzyme or does not absorb at a unique wavelength, there are still suitable assays (for example mass spectrometry) for identifying the product. The inventors chose to monitor the purification of rBoNT(H_C) through immunological
10 detection by Western blot analysis. However, with various polyclonal antibodies against whole toxin available but without an appropriate positive control, the Western blot results can only be interpreted as ambiguous until a purified sample is sequenced or shown to be protective.

 There are two major issues of concern when extracting C-fragment, H_N,
15 and/or heavy chain (H_C) antigens from pichia cells. The first concern is the solubility of these proteins (i.e., can enough product be extracted into the soluble fraction for further processing?). The second concern deals with the effective removal of polynucleic acids and/or other contaminating materials, which strongly interfere with the necessary chromatography.

20 The zwitterionic detergent, CHAPS, is most notably an effective agent for solubilizing membrane proteins. Membrane proteins exist in a hydrophobic environment, and if removed from that environment, possess strong tendencies to aggregate and ultimately precipitate. CHAPS prevents that aggregation from occurring with membrane bound proteins. The inventors extrapolated this premise
25 to the clostridial proteins noted above. C-fragments, translocation domains (H_N), and entire heavy chains are missing their natural partner (the remaining segments of the neurotoxin) and thus, presumably bare exposed hydrophobic regions on their protein surface where the H_C, H_N, or heavy chain normally associates with rest of the neurotoxin. These exposed hydrophobic regions are potential nucleation sources
30 for protein aggregation, because the natural tendency of a protein in an aqueous environment is to bury their hydrophobic surface. When pichia cells are disrupted with CHAPS (on the order of 0.3% W/V) present in the cracking buffer, the amount

of fragment C protein isolated in the soluble fraction has been observed to increase from less than 5% to nearly 80% with serotype C₁. Dramatic increases in solubility have been noted with C-fragment serotypes A and F as well.

Once a soluble antigen has been produced, the subsequent task is to separate
5 that antigen from the myriad of pichia host proteins, lipids, and other impurities that exist in the extracted medium. In order for the chemical separations to be feasible by liquid chromatography, it is critical that polynucleic acids be efficiently removed. Nucleotides will either bind to the C-fragment (serotypes A, E, and F due to their elevated pIs) or will bind to the anion-exchange chromatography resin (as is used in
10 the first purification step of the C₁ process). With either case, the chromatography is rendered futile. The C-fragment product will either fail to bind to the chromatography media or it will elute over an unacceptably large sodium chloride concentration range. Pichia cells possess an abundant amount of DNA. Polyethyleneimine (PEI) is a polycationic agent that readily precipitates nucleotides.
15 When pichia cell extracts are treated with PEI, the nucleic acids are efficiently precipitated and removed by centrifugation without significant loss of product. More importantly, the chromatographic separation of C-fragments from pichia proteins are dramatically improved.

The soluble portion of the cell lysate may typically be purified in two
20 conventional chromatographic steps. The ultimate objective of this work is to obtain FDA licensure of rBoNT as a safe and effective vaccine. Even though separations can be accomplished at extremely high resolution with affinity chemistry, there remains an undesirable effect of hapten leaching from the resin. Thus, a preferred separation employs a cation-exchange step followed by hydrophobic interaction
25 chromatography (HIC). These two steps complement each other as they provide separations based on electrostatic and hydrophobic interactions. The cation-exchange step was particularly efficient in increasing the purity of rBoNTF(H_C), as the antigen was estimated to be purified greater than 52-fold. The efficiency of purification is primarily attributed to the significant difference in isoelectric points
30 between most pichia proteins (pIs<7) and rBoNT(H_C) (experimental pI=9.4 for rBoNTF(H_C) , data not shown) and thus, the pichia proteins were removed in the column flow through. Precipitate that results when the cation-exchange pool is

treated with ammonium sulfate contains mostly pichia proteins and very little rBoNT product. The HIC step removes most or all of the remaining impurities. The yield of soluble rBoNTF(H_C) from the total recombinant yeast cell lysate was estimated to be greater than 28% with a purity greater than 98%. Use of similar
5 purification steps for rBoNTA(H_C) produced greater than 95% pure material.

A significant amount of rBoNTF(H_C) product (30-40%) was identified in the insoluble portion of the cell lysate. Also, the antigen was 35% of the total protein present in the pellet; in effect it was more pure than the soluble rBoNTF(H_C) was after the ion-exchange step. This suggests an alternative process whereby insoluble
10 rBoNT product produced in yeast may be resolubilized and purified to homogeneity. The resolubilization may be performed by resuspending the pellet in urea and subsequently removing the urea by dialyses in nondenaturing buffer. A single chromatographic step using cation-exchange chemistry may be sufficient to purify the resolubilized antigen, in some cases to greater than 98%. The yield of
15 resolubilized rBoNTF(H_C) product from the total cell lysate was estimated to be >19%. The overall bench scale yield of purified soluble and resolubilized rBoNTF(H_C) was estimated to be greater than 47% or 240 mg/Kg of the cell paste. A similar procedure would be suitable for purification of rBoNTA(H_C) and other rBoNT fragment peptides from yeast.

20 Analysis of CD spectra of both soluble and resolubilized product revealed the presence of significant β -sheet which is in agreement with that predicted for rBoNTF(H_C) using an artificial neural network (Lebeda, F.J., et al., (1997), "Predicting Differential Antigen-Antibody Contact Regions Based on Solvent Accessibility," *J. Protein Chem.*, **16**:607-618), and that determined by crystal
25 structure of BoNT serotype A (Lacy, D.B., et al., (1998), "Crystal Structure of Botulinum Neurotoxin Type A and Implications for Toxicity," *Nat. Struct. Biol.*, **5**:898-902). However, even though CD revealed that the two antigens possessed similar folds, there were subtle differences between the two spectra suggesting that the secondary structures, and hence, tertiary structures were not identical.

30 Immunization

The purified soluble and resolubilized antigens appear to be in a folded conformation. However, the bottom line with any potential vaccine is the

demonstration of protection. Are the antigens in a conformation that will elicit the production of neutralizing antibody? To answer this question, mice were inoculated with rBoNTF(H_C) and subsequently challenged with a high level of rBoNTF toxin. The purified soluble rBoNTF(H_C) completely protected mice receiving three
5 inoculations of 0.2 µg from challenge with 1000 mouse i.p. LD₅₀ of BoNT/F toxin. Analysis of the association of dose with survival indicated that dose was associated with the odds of surviving (odds ratio=2.0, meaning that the odds of survival increase twofold per unit increase in dose with a 95% confidence level from 1.3 to 3.1). The number of inoculations was also associated with survival. Both two
10 inoculations and three inoculations were associated with increased odds of survival relative to a single inoculation (5.3-fold with a 95% confidence level of 1.2-23 for two inoculations and 22-fold with a 95% confidence level of 4.3 -110 for three inoculations). It is apparent that a single shot at higher doses achieved protection comparable to multiple inoculations at lower doses. Also, three doses of 1 µg of
15 purified resolubilized rBoNTF(H_C) completely protected mice from a challenge of 5000 mouse i.p. LD₅₀ of BoNTF toxin, thus demonstrating that refolded rBoNTF(H_C) from the insoluble fraction of lysate could also be a prosperous source of antigen.

Individual antibody ELISA titer appears to be an excellent predictor of
20 mouse survival. If the antibody titer of a mouse was 100 or greater, that mouse was predicted to and did survive a challenge of 1000 mouse i.p. LD₅₀ of BoNTF toxin. Upon vaccination of mice with 2 or 3 doses of rBoNTA(H_C) or rBoNTB(H_C) vaccine delivered on a specific schedule (i.e., parental intramuscular injection at 0,4, and 8 weeks), survival of animals challenged with 100,000 or 1,000,000 million
25 LD₅₀ of toxin is very high. Measurement of the antibody levels in these animals via an ELISA shows that the survival rate can be correlated with the measured antibody level. The ELISA is performed by coating a microtiter plate with toxin or fragment C itself, then sera from the vaccinated mice is added at various dilutions (i.e., sera diluted 1/100, 1/400, 1/1600, 1/6400, etc.). Since fragment C is sufficient to elicit
30 protection in animals, preferably assays for neutralizing antibody titer in sera from animals vaccinated with fragment C are performed using microtiter plates coated with fragment C. Antibody in the sera will bind to the toxin or the fragment C, and the

bound antibody may be detected by a secondary antibody (e.g., anti-mouse IgG) that is coupled to horse-radish peroxidase or alkaline phosphatase. The secondary IgG will bind to the anti-BoNT antibody that was raised to the fragment C vaccine. After washing the microtiter wells, a substrate for the peroxidase or phosphatase enzymes is added to the wells. The substrate will give off a color once the enzyme has cleaved the substrate, and the intensity of the color measured (e.g., at 405 nm. Typically, a reading of 0.2 is used as the base. Thus, if dilution of the sera by 1/1600 gives a reading of 0.15 at 405 nm and a dilution of 1/400 gives a reading of 0.45 at 405 nm, the antibody titer in the sera is characterized as 1/400 dilution (i.e., titer of 400 fold). Obviously, if readings of 0.2 are obtained at higher dilutions, better protection is observed. With rBoNTA(Hc) vaccination, for mice which had ELISA titers of less than 100, only 14.3% survival rate was observed under the conditions of vaccination and challenge. With rBoNTF(Hc), for mice which had ELISA titers of 100 fold, under the condition of vaccination and challenge, 100% of the mice were protected.

It also will be well known to one of ordinary skill in the art that a susceptible host may be immunized using the appropriate peptide vaccine formulated in adjuvant to increase the immune response. Such adjuvants include but are not limited to Freund's (complete and incomplete), mineral gels, such as aluminum hydroxide, surface active substances such as keyhole limpet hemocyanin, lysolecithin, pluronic polyols, polyanions, peptides, BCG (Bacille Calmette-Guerin), oil emulsions and dinotrophenols. Immunization can be carried out with additional various presentation and cross-linking permutations. By way of example and not of limitation, such permutations include rBoNT peptides cross-linked to KLH as a carrier, any rBoNT peptide cross-linked to any other rBoNT protein as carrier, rBoNT peptides cross-linked to themselves, and these combinations presented by the various adjuvants listed above. It will become evident that such permutations are available in regard to other peptides and self-assembled peptides disclosed throughout this specification.

It will also be known to one of ordinary skill in the art that use of the term "susceptible host" includes any such mammalian host susceptible to intoxication by BoNT. It will be further evident that any such susceptible host is a candidate for

treatment to promote protection from BoNT utilizing the peptide vaccines and associated methods described in this specification.

EXAMPLES

5 In order to facilitate a more complete understanding of the invention, a number of Examples are provided below. However, the scope of the invention is not limited to specific embodiments disclosed in these Examples, which are for purposes of illustration only.

10 Example 1. Synthesis and cloning of a synthetic gene encoding rBoNTF(H_C)

A synthetic gene encoding a putative fragment C region of botulinum neurotoxin serotype F was designed and constructed for expression in *Escherichia coli* (Holley *et al.*, submitted to Vaccine). The recombinant BoNTF(H_C)₁ gene was expressed in *E. coli* as a fusion protein with maltose-binding protein (MBP) with yields of 1 mg/L culture (See Figure 18).

The same gene was used for expression studies in the yeast, *P. pastoris*. This particular host was chosen because it could produce high levels of recombinant proteins (Cregg, J.M., et al., (1993), "Recent Advances in the Expression of Foreign Genes in *Pichia pastoris*," *Bio/Technology*, 11:905-909; Romanos, M.A., et al., 20 (1992), "Foreign Gene Expression in Yeast: A Review," *Yeast*, 8:423-488; Sreekrishna, K., et al., (1988), "High Level Expression of Heterologous Proteins in Methylophilic Yeast *Pichia pastoris*," *J. Bas. Microbiol.*, 28:265-278) and because it lacked endotoxins which would facilitate product development. Intracellular expression of the antigen was used to avoid potential glycosylation of the 25 recombinant protein. The rBoNTF(H_C)₁ gene was modified at its 3' end for insertion into the unique EcoR I site of the yeast vector, pHILD4. The recombinant construct containing the rBoNTF(H_C)₁ gene was subsequently linearized with Sac I and the cassette integrated into the chromosomal alcohol oxidase (AOX 1) of *Pichia pastoris* strain GS115 (Clare, J.J., et al., (1991), "High-Level Expression of Tetanus 30 Toxin Fragment C in *Pichia pastoris* Strains Containing Multiple Tandem Integrations of the Gene," *Bio/Technology*, 9:455-460). Yeast transformants expressing the selectable markers histidine dehydrogenase (Cregg, J.M., et al.,

(1985), "*Pichia pastoris* as a Host System for Transformations," *Mol. Cell. Biol.*, 5:3376-3385) and aminoglycoside phosphotransferase 3' (I) (Scorer, C.A., et al., (1994), "Rapid Selection Using G418 of High Copy Number Transformants of *Pichia pastoris* for High-Level Foreign Gene Expression," *Bio/Technology*, 12:181-184) were isolated. These isolates were further characterized for their ability to express rBoNTF(H_C) after induction with methanol. Although the various transformants generated were able to express the selectable markers, no expression of rBoNTF(H_C) as judged by SDS/PAGE and blot analysis was observed in these isolates (data not shown).

10 **SDS/PAGE, Western blot, and protein assays**

Total protein concentrations were determined by using the Pierce BCA™ (bicinchoninic acid) protein assay kit with BSA as a standard. The purity of the rBoNTF(H_C) product was assessed by SDS/PAGE with Novex (San Diego, CA, U.S.A.) gel electrophoresis supplies, reagents, protocols, and National Institutes of Health (NIH) imaging software as previously described (Byrne, M.P., et al., (1998), "Purification, Potency, and Efficacy of the Botulinum Neurotoxin Type A Binding Domain from *Pichia pastoris* as a Recombinant Vaccine Candidate," *Infect. Immun.*, 66:4817-4822). Western blot assays were used to identify FPLC fractions containing rBoNTF(H_C) as previously described (Byrne, 1998) with the following changes. The primary antibody used was a polyclonal protein G sepharose-purified horse anti-BoNTF antibody incubated at 1 µg/ml for 3 h. The secondary antibody used was a horseradish peroxidase-labeled affinity-purified goat anti-horse IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD, U.S.A.) assayed at 1 µg/ml for 2 h.

25 **Example 2. Synthesis and cloning of a synthetic gene encoding rBoNTF(H_C)**

A second synthetic gene, rBoNTF(H_C)₂, was subsequently designed to facilitate expression in *P. pastoris*. Redesigning the gene was intended to lower specific regions of the rBoNTF(H_C)₁ gene in which spikes of AT-rich tracts still remained. Previous work had shown that rare codons (Makoff, A.J., et al., (1989), "Expression of Tetanus Toxin Fragment C in *E. coli*: High Level Expression by Removing Rare Condons," *Nucleic Acids Res.*, 17:10191-10201) and/or highly enriched AT base compositions (Romanos, M.A., et al., (1991), "Expression of

Tetanus Toxin Fragment C in Yeast: Gene Synthesis is Required to Eliminate Fortuitous Polyadenylation Sites in AT-rich DNA," *Nucleic Acids Res.*, **19**:1461-1467) in clostridial DNA were incompatible with optimum expression of clostridial genes in *E. coli* and yeast. A second synthetic gene encoding the rBoNTF(H_C) fragment was designed and constructed using *P. pastoris* codon usage (Sreekrishna, K., (1993), "Strategies for Optimizing Protein Expression and Secretion in the Methylophilic Yeast *Pichia pastoris*," *Industrial Microorganisms: Basic and Applied Molecular Genetics*, (Baltz, R. H., et al, Eds.), pp. 119-126, Am. Soc. Microbiol., Washington, DC). Briefly, complimentary oligonucleotides encoding the amino terminal region of the F(H_C) (423 nucleotides flanked with *EcoRI* and *PstI* sites), the central region of the F(H_C) (606 nucleotides flanked by *PstI* and *SalI* sites) and the carboxy-terminal region of F(H_C) (336 nucleotides flanked by *SalI* and *EcoRI* sites) were annealed and cloned into pUC or PCR zero-blunt plasmid vectors. The AT base composition in the native clostridial F(H_C) DNA averaged 76% while rBoNTF(H_C)₁ averaged 58% and rBoNTF(H_C)₂, 53% (Figure 19). The synthetic gene sequence of rBoNTF(H_C)₂ and the 432 amino acids it encoded for is shown in Figure 9. After nucleotide sequencing, the cloned fragments were excised by the appropriate restriction endonucleases, separated by agarose gel electrophoresis, and purified. The isolated DNA fragments were ligated simultaneously into *EcoR* I digested and dephosphorylated plasmid pHIL4. The vector harboring the rBoNTF(H_C)₂ gene was integrated into the chromosomal *AOX1* locus of *P. pastoris* as described above. Transformants expressing selectable markers (histidine dehydrogenase and aminoglycoside phosphotransferase 3' (I)) were isolated and tested for their ability to express rBoNTF(H_C). Unlike the rBoNTF(H_C)₁ gene, rBoNTF(H_C)₂ was expressed after induction with methanol and yielded the expected molecular weight of approximately 50000 daltons as judged by SDS/PAGE and Western blot analysis (Figure 20). The deduced molecular mass of the encoded polypeptide was 50,250 daltons.

Example 3. Expression and Cell Disruption of rBoNTF(H_C) in *P. pastoris*

Large-scale fermentation conditions and optimal intracellular expression of rBoNTF(H_C) were determined for the yeast strain *P. pastoris*.

Protein expression

A stock seed culture of *P. pastoris* was grown in shake-flasks containing 0.5 L of YNB medium (13.4 g/L yeast nitrogen base without amino acids, 20 g/L glycerol, 0.4 mg/L biotin, in 100 mM sodium phosphate, pH 6.0). Cultures were grown at 30°C until an A₆₀₀ of 20 absorbance units was achieved, and then used to inoculate a 5-L BioFlo 3000 fermentor (New Brunswick Scientific, Edison, NJ, U.S.A.) containing 2.5 L basal-salt medium plus PTM₄ trace mineral salts and 4% glycerol. Dissolved oxygen was maintained at 40% and the pH was maintained at 5.0 with 30% ammonium hydroxide. After the initial glycerol was consumed, 50% (w/v) glycerol was added at a rate of 20 g/L/h for 1 h then decreased linearly to 0 g/L/h over 3 h. The medium was enriched with 1.5 g methanol/L of medium. Methanol feed was started at 4 g/L/h and linearly increased to 9 g/L/h over 10 h. The methanol feed rate was adjusted by using the dissolved oxygen-spike method (Chiruvolu, V., et al., (1997), "Recombinant Protein Expression in an Alcohol Oxidase-Defective Strain of *Pichia pastoris* in Feed-Batch Fermentations, *Enzyme Microbiol. Technol.*, **21**:277-283). After 10 h of methanol induction, the cells were harvested by centrifugation at 6000 g for 10 min at 4°C with a Beckman JA-10 rotor (Beckman Instruments, Palo Alto, CA, U.S.A.) and then stored at -20°C.

Protein expression

A stock seed culture of *P. pastoris* was grown in shake-flasks containing 0.5 L of YNB medium (13.4 g/L yeast nitrogen base without amino acids, 20 g/L glycerol, 0.4 mg/L biotin, in 100 mM sodium phosphate, pH 6.0). Cultures were grown at 30°C until an A₆₀₀ of 20 absorbance units was achieved, and then used to inoculate a 5-L BioFlo 3000 fermentor (New Brunswick Scientific, Edison, NJ, U.S.A.) containing 2.5 L basal-salt medium plus PTM₄ trace mineral salts and 4% glycerol. Dissolved oxygen was maintained at 40% and the pH was maintained at 5.0 with 30% ammonium hydroxide. After the initial glycerol was consumed, 50% (w/v) glycerol was added at a rate of 20 g/L/h for 1 h then decreased linearly to 0 g/L/h over 3 h. The medium was enriched with 1.5 g methanol/L of medium. Methanol feed was started at 4 g/L/h and linearly increased to 9 g/L/h over 10 h. The methanol feed rate was adjusted by using the dissolved oxygen-spike method (Chiruvolu, 1997). After 10 h of methanol induction, the cells were harvested by

centrifugation at 6000 x g for 10 min at 4°C with a Beckman JA-10 rotor (Beckman Instruments, Palo Alto, CA, U.S.A.) and then stored at -20°C.

Cell disruption and sample preparation

Eleven g of frozen cell paste was resuspended in 100 ml of 50 mM Na₂HPO₄/2 mM Na₂EDTA/1 mM PMSF, pH 6.8 at 4°C. The suspended cells were
5 disrupted by three successive passes through a microfluidizer device (model 110Y, Microfluidics Corp., Newton, MA, U.S.A.) at 21000 psi. The temperature of the disruptate was kept below 10°C throughout the process by cooling the exit line and collection flask with ice. The cells were judged to be greater than 95% disrupted as
10 determined by microscopy. In comparison, 8-10 passes through a Gaulin homogenizer were required to efficiently disrupt the cells in past protocols. SDS-PAGE and Western blot analysis of cell lysate showed that expressed rBoNTF(H_C) represented <0.5% of the total protein. The resulting cell lysate volume was 105 ml with a protein concentration of 11 mg/ml. Cellular debris and insoluble proteins
15 were removed by centrifugation at 15000 g for 15 min at 4°C with a Sorval SS-34 rotor (Sorval Instruments, Newtown, CT, U.S.A.). The resulting extract was noticeably turbid due to the presence of lipids and significant quantities of nucleic acids. As rBoNTF(H_C) possessed a calculated isoelectric point of 9.1 and presumably interacted strongly with DNA, DNase was added to the cell extract in
20 order to digest the polynucleotides and facilitate purification. To remove the polynucleotides, the extract was treated with DNase (100 units/ml, Aldrich) and ZnCl₂ (2 mM, Aldrich) at room temperature for 30 min and then dialyzed extensively with 10 kDa molecular weight cut off (MWCO) Slide-A-Lyzer dialysis cassettes (Pierce) in 50 mM Na₂HPO₄/2 mM Na₂EDTA/1 mM PMSF, pH 6.8 at 4°C.
25 A precipitate developed during dialysis that was separated by centrifugation at 15000 g for 15 min at 4°C with a Sorval SS-34 rotor. The clarified extract contained 7.8 mg/ml of total protein and was used as starting material for the FPLC purification of soluble rBoNTF(H_C) while the pellet was used as starting material for the resolubilized rBoNTF(H_C) purification.

Example 4. Conventional Purification of rBoNTF(H_C) from *P. pastoris*

The rBoNTF(H_C) protein was purified to homogeneity using an FPLC system and two chromatographic steps. First, the material was subjected to cation exchange chromatography (Figure 21A).

5 FPLC purification of soluble rBoNTF(H_C)

Soluble rBoNTF(H_C) was purified by using a Pharmacia model 500 FPLC system (Pharmacia, Uppsala, Sweden) with programmed elution and A₂₈₀ monitoring. The starting material was loaded onto a Pharmacia HR 10/10 Mono S cation-exchange column equilibrated with 50 mM Na₂HPO₄/2 mM Na₂EDTA/1 mM
10 PMSF, pH 6.8 (buffer A) at a flow rate of 2 ml/min (150 cm/h). The column was washed with 16 ml (2 bed volumes) of buffer A. Flow through and wash were collected separately and stored for subsequent analysis. Protein was eluted from the column with a linear gradient from 0 to 300 mM NaCl over 80 ml (10 bed volumes), then a linear gradient from 300 to 1000 mM NaCl over 20 ml (2.5 bed volumes), and
15 then an isocratic gradient at 1000 mM over 10 ml (1.25 bed volumes). Four-ml fractions were collected throughout the linear and isocratic gradients. This step was highly efficient as most pichia proteins possess isoelectric points between pH 5 and 7 and, therefore, pass through the column without binding. Fractions eluting between 230 and 260 mM NaCl were positive for rBoNTF(H_C) by Western blot
20 analysis and were pooled. The pooled fractions were adjusted to 1.5 M ammonium sulfate by the slow addition of 2 M (NH₄)₂SO₄/50 mM Na₂HPO₄/2 mM Na₂EDTA/25 mM NaCl, pH 7.5 with stir bar agitation. A protein precipitate formed which consisted primarily of yeast proteins with a small amount of rBoNTF(H_C) product (approximately 10%). The precipitate was removed by centrifugation at
25 6000 g for 10 min at 4°C with a Sorval SS-34 rotor. Fortunately, when the pool of Mono S column fractions was diluted with ammonium sulfate, most of the rBoNTF(H_C) product remained in solution (approximately 90%) while significant quantities of pichia proteins salted out. The first step enriched the desired product from <0.5 to 26% of the total protein (Table 2).

30 HIC was used as a second chromatographic step (Figure 21B) and separated proteins based on their differences in surface hydrophobicity. It was determined that neopentyl chemistry provided the appropriate hydrophobic interaction with

rBoNTF(H_C). The supernatant was loaded onto a Pharmacia alkyl superose 10/10 hydrophobic interaction chromatography (HIC) column equilibrated with 1.5 M (NH₄)₂SO₄/50 mM Na₂HPO₄/2 mM Na₂EDTA/25 mM NaCl, pH 7.5 (buffer B) at a flow rate of 1 ml/min (75 cm/h). The column was washed with 8 ml (1 bed volume) of buffer B. Protein was eluted from the column with a linear gradient of decreasing (NH₄)₂SO₄ from 1.5 to 0 M over 60 ml (7.5 bed volumes). The rBoNTF(H_C) eluted from the HIC column at 0.92 M ammonium sulfate in a volume of 3 ml with a protein concentration of 0.52 mg/ml. Fractions positive by Western blot analysis and which only showed a single band by SDS/PAGE were pooled and dialyzed extensively in 50 mM Na₂HPO₄/2 mM Na₂EDTA, pH 6.8.

The recovery of purified product from cell extract was estimated to be greater than 42%, with a yield of 140 mg/kg of cell paste (Table 2). The resulting rBoNTF(H_C) was judged to be greater than 98% pure as only a single band was detected by SDS-PAGE (Figure 20) even when moderately (4 μg) overloaded. Capillary isoelectric focusing showed the antigen possessed an isoelectric point of 9.4 (data not shown), which is in reasonable agreement with the calculated pI of 9.1.

TABLE 2. Purification of soluble rBoNTF(H_C)

Total protein concentration was determined by Pierce BCA™ assay. rBoNTF(H_C) was identified by Western blot analysis and purity was estimated by analysis of individual lanes of SDS/PAGE by pixel densitometry using NIH imaging software.

Step	Concentration (mg/ml)	Protein (mg)	rBoNTF(H _C) (mg)	Purity (%)	Fold Purification	Recovery (%)
Lysate	11	1100	5.6	<0.5	--	3.8
Dialzed extract	7.8	740	3.7	<0.5	--	66
Mono S	1.2	9.6	2.5	26	>52	45
Alyl superose	0.52	1.6	1.6	100	3.8	29

CD of purified soluble and resolubilized rBoNTF(H_C)

Purified soluble and resolubilized rBoNTF(H_C) were subjected to CD spectroscopy in a Jasco 600 spectropolarimeter (Japan Spectroscopy company, Tokyo, Japan). Experiments were performed at a concentration of 30 μg/ml (0.62 μM) in a 1 cm path length cell in 10 mM Na₂HPO₄, pH 7.0. Spectra were obtained

as an average of four accumulations, scanned from 260-200 nm, at a scan rate of 10 nm/min, with a 2 sec response, and a 1 nm band width. The temperature was maintained at 20°C with a Peltier thermocontrol device.

Analysis of the far-UV circular dichroism spectrum (Figure 22) of the purified antigen showed a positive peak at 233 nm and a minimum at 214 nm. This suggests the molecule is in a folded conformation and possesses considerable β -sheet.

Example 5. Purification of Resolubilized rBoNTF(H_C)

Western blot analysis revealed that approximately 30-40% of the total expressed rBoNTF(H_C) was present in the insoluble pellet after cell lysis. To investigate whether this insoluble protein could be recovered, the pellet was extracted in the denaturant urea and then dialyzed in nondenaturing buffer.

FPLC purification of resolubilized rBoNTF(H_C)

The cell lysate pellet was resuspended into 20 ml of 3 M urea/50 mM Na₂HPO₄, pH 7.0 and extracted 15 h at 4°C on a Labquake rotator. The cellular components not solubilized by the denaturing buffer were removed by centrifugation with a Sorval SS-34 rotor at 15000 g for 10 min at 4°C. The supernatant was dialyzed extensively using 10 kDa MWCO Pierce Slide-A-Lyzer dialysis cassettes in buffer A. A slight precipitate formed during the dialysis which was removed by centrifugation as described above. Western blot analysis showed that rBoNTF(H_C) was present only in the supernatant, which was estimated to be about 35% pure by SDS/PAGE. The supernatant was loaded onto a Pharmacia HR 10/10 Mono S cation-exchange column and separated by the same conditions as above. Fractions containing only a single positive rBoNTF(H_C) band SDS/PAGE and Western blot analysis were pooled and dialyzed in 50 mM Na₂HPO₄/2 mM Na₂EDTA, pH 6.8 in 10 kDa MWCO dialysis cassettes. The final resolubilized rBoNTF(H_C) product was judged to be greater than 98% pure as determined by SDS/PAGE.

After a single cation exchange chromatography separation step, the rBoNTF(H_C) was greater than 98% pure as judged by SDS-PAGE. The total yield of purified resolubilized rBoNTF(H_C) was 100 mg/kg of cell paste. The conformation of purified resolubilized antigen showed significant β -sheet as determined by CD spectral analysis (Figure 22). However, the overall fold appeared

slightly different than that shown by rBoNTF(H_C) purified from the cell lysate supernatant. The primary difference was the lack of a positive peak at 233 nm, indicating differences in β -sheet content.

Example 6. Mouse Immunogenicity and Efficacy Studies

5 To assess the immunogenicity of the recombinant rBoNTF(H_C), mice were inoculated with either one, two, or three doses of purified rBoNTF(H_C) from the soluble fraction of lysate at doses ranging from 0.008 to 5 μ g per mouse.

Mouse inoculations and BoNTF toxin challenge

Mice, Crl:CD-1, ICR mice (Charles River, NC, U.S.A.) weighing 16-22 g on receipt, were injected intramuscularly (i.m.) with purified rBoNTF(H_C). Mice were challenged intraperitoneally (i.p.) 21 days after their last rBoNTF(H_C) injection with BoNTF toxin complex (Langeland strain) diluted in .0.2% (w/v) gelatin/0.4% (w/v) Na₂HPO₄, pH 6.2, in 100 μ l total volume per mouse. Groups of five naive mice were also used as toxin controls. Mice were observed daily and deaths were recorded five days post challenge. All animal manipulations were in accordance with applicable regulations in AAALAC-accredited facilities.

The efficacy of the purified soluble rBoNTF(H_C) was determined by inoculating groups of five female mice with one, two, or three doses of 0.008, 0.04, 0.2, 1.0, or 5.0 μ g rBoNTF(H_C) (diluted in 100 μ l of 0.2% (v/v) Alhydrogel (Superfos Biosector, Kvistgaard, Denmark) in 0.9% (w/v) saline) per mouse at 14 day intervals. Two days before challenge, mice were bled retroorbitally and serum was collected for ELISA testing. Mice were challenged with 1000 mouse i.p. LD₅₀ of BoNTF toxin complex.

All of the mice, including five naïve controls, were challenged with 1000 mouse ip LD₅₀ of BoNTF toxin. The controls all died within 2-4 h. A dose response was observed from groups of mice receiving different numbers of inoculations (Table 3). A single inoculation of 5 μ g protected four of five mice, while a dose of 0.2 μ g or below protected one or no mice. Two and three inoculations protected four of five and five of five mice at doses of 0.2 and 0.04 μ g, respectively. At all dose levels studied, the number of surviving mice increases with the number of inoculations.

Serum antibody titers for each individual mouse were determined by ELISA, followed by calculation of the geometric mean titers for each group in the study.

Mouse serum ELISA

Individual mouse serum ELISAs were performed as previously described (Byrne, 1998) except for the following differences. Botulinum neurotoxin serotype F (Langeland strain, Food Research Institute, University of Wisconsin, Madison, WI, U.S.A.) was used as the coating antigen and the positive control for each assay was a mouse IgG monoclonal antibody, 7F8.G2.H3 (Brown, D.R., et al., (1997), "Identification and Characterization of a Neutralizing Monoclonal Antibody Against Botulinum Neurotoxin, Serotype F, Following Vaccination with Active Toxin," *Hybridoma*, **16**:447-456).

TABLE 3. Survival, antibody group ELISA titers, and serum neutralization titers of mice after inoculation with purified soluble rBoNTF(H₂)

Mice were challenged with 1000 i.p. LD₅₀ BoNTF toxin 21 days after last inoculation. Antibody ELISA titers were measured as the reciprocal of the highest dilution having an OD₄₀₅ greater than 0.2 AU after correcting for background. Geometric mean ELISA titers were determined by taking the geometric mean of the logarithm of the individual titers. Standard deviations of the geometric means are also reported. If the ELISA titer was determined to be below the detection limit of the assay (<100), the ELISA titer was arbitrarily assigned a value of 25. A geometric mean titer value of 1.4 means that all ELISA titers within that group were below the detection limit.

Vaccination	Survival (alive/5 tested)			Geometric mean ELISA titers		
dose (μg)	1X	2X	3X	1X	2X	3X
0.008	0*	0	2	1.4	1.4	1.6±0.3
0.04	0	1	4	1.4	1.5±0.3	2.4±0.8
0.2	1	4	5	1.4	2.1±0.9	2.9±1.3
1.0	2	5	5	1.4	2.8±0.3	4.3±0.3
5.0	4	4	5	1.6±0.3	2.8±0.9	4.1±0.5

* Only four mice were tested within this group

Statistical analysis

The logistic regression model was used to test associations of geometric mean ELISA titers and individual titers with survival by using SAS, version 6.10. Geometric mean titers correlated well with protection (Table 3). The three groups with no survivors had geometric means titers below the detection limit of the assay (1.4). Similarly, the four groups that showed complete protection had geometric means titers of 2.8 or greater. Individual mouse antibody titers correlated extremely well with protection (Table 4). Only 7 out of 38 mice survived whose titers were below 100. On the other hand, 34 out of 34 survived whose titers were 100 or greater. One mouse in the study could be classified as a "nonresponder." The mouse, receiving two injections at the highest dose level, had an antibody titer below the detection limit and did not survive the BoNTF challenge. The rest of the mice in that particular group had titers of 1600 or greater.

15

TABLE 4. Correlation of individual antibody ELISA titer with protection after inoculation with purified soluble rBoNTF(H_c)

Serum was bled from each mouse individually. Titer is reciprocal of the highest dilution having an OD₄₀₅ greater than 0.2 AU after correcting for background. Mice were challenged with 1000 i.p LD₅₀ BoNTF toxin 21 days after last inoculation.

Individual ELISA titer	Survival (alive/total)*	% survival
<100	7/38	18.4
100	7/7	100
400	4/4	100
1600	11/11	100
6400	3/3	100
25600	9/9	100

* The individual antibody titers from three mice were not measured. Two mice did not offer enough serum and one mouse was not challenged.

The resolubilized antigen was also evaluated for immunogenicity and protective efficacy by its ability to protect mice from a BoNTF toxin challenge. Groups of 10 male mice each received three inoculations of either 1 μ g or 5 μ g of rBoNTF(H_C) (diluted in 100 μ l 0.2% (v/v) Alhydrogel in 0.9% (w/v) saline) per mouse at 14 day intervals. Two days before challenge, mice were bled retroorbitally and serum was collected for ELISA testing. Mice inoculated with 1 μ g doses were challenged with 5000 mouse ip LD₅₀ of BoNTF toxin. Ten of ten mice survived the challenge. Because 100% protection was observed with the group inoculated with 1 μ g doses, the group that received three doses of 5 μ g were subjected to a challenged level two orders of magnitude greater in order to test the limits of the antigen. Therefore, the 5 μ g dose group was challenged with 500,000 mouse ip LD₅₀ of BoNTF toxin. None of the mice survived the challenge; however, a significant delay in time to death was observed (24-48 h). All the control mice succumbed within 2-4 h after challenge.

Example 7. Synthesis and cloning of a synthetic gene encoding rBoNTA (H_C)

The preparation of genetically engineered proteins to provide protection from the toxins produced by *Clostridium botulinum* was accomplished in *E. coli*.

Restriction endonucleases and DNA modifying enzymes were obtained from GIBCO BRL (Gaithersburg, Maryland). Polymerase chain reaction (PCR) reagents were purchased from Perkin-Elmer Cetus (Norwalk, CT). SDS PAGE precast gels and running buffers were acquired from Amersham (Arlington Heights, Illinois). All oligonucleotides were synthesized by Macromolecular Resources (Ft. Collins, Colorado). ELISA reagents were obtained in house or from Sigma (St. Louis, Missouri) or Kirkegard and Perry Laboratories (Gaithersburg, Maryland).

The *Escherichia coli* host was K12DH5a, purchased as competent cells from GIBCO BRL. Expression vectors pMAL from New England Biolabs (Beverly, Massachusetts) and pKK233-2 from Pharmacia LKB (Piscataway, New

Jersey) were used according to the manufacturers' standard protocols. The DNA clone coding of the H_C domain of *C. botulinum* toxin serotype A was pCBA3, kindly provided by Nigel Minton.

Oligonucleotide primers incorporating appropriate terminal
 5 restriction enzyme sites were used to PCR amplify the H_C region of the *C.*
botulinum clone pCBA3. Gel-purified insert DNA and vector DNA were cleaved
 with the appropriate restriction enzymes, purified on low melting point agarose, and
 ligated overnight at room temperature. Competent DH5a host cells were
 transformed according to suppliers recommendations and plated on LB plates with
 10 100 ug/ml ampicillin. Protein electrophoresis was run on precast 11-20% SDS
 PAGE at the manufacturer's recommended parameters. ELISA plates were
 incubated with capture antibody (horse anti-botulinum A polyclonal serum)
 overnight, then blocked with skim milk prior to application of various dilutions of
 test material, signal antibody (rabbit antitoxin A polyclonal serum), signal HRP
 15 conjugated anti-(rabbit IgG) and ABTS substrate solution. Plates were read on an
 automated reader at 405 nm.

The sequence of the C fragment of the A chain was deduced as:

RYESNHLIDLSRYASKINIGSKVNFDPIDKNQIQLFNLESSKIEVILKNA
 IVYNSMYENFSTSFWIRIPKYFNSISLNNEYTIINCMENNSGWKVSLSNYGEII
 20 WTLQDTQEIQRVVFVKYSQMINISDYINRWIFVTITNNRLNNSKIYINGRLID
 QKPISNLGNIHASNNIMFKLDGCRDTHRYIWIFYFNLFDKELNEKEIKDLYDN
 QNSGILKDFWGDYLQYDKPYMLNLYDPNKYVDVNNVGIRGYMYLKGP
 RGSVMTTNIYLNSSLYRGTKFIKKYASGNKDNIVRNNDRVYINVVKNKE
 YRLATNASQAGVEKILSALEIPDVGNLSQVVVMKSKNDQGITNKCKMNLQ
 25 DNNGNDIGFIGFHQFNNAKLVASNWNQRQIERSSRTLGCSEFIPVDDGWG
 ERPL

The C fragment protein sequence was reverse translated using *E. coli* optimal
 codon usage. The gene was then altered in many places to insert restriction sites,
 start codon, stop codon. Other changes were also effected to make the molecule
 30 more appropriate for use in the vector. Throughout, the fidelity of the protein
 sequence generated therefrom was maintained.

The sequence for the synthetic gene is found below:

CTCGAGCCATGGCTCGTCTGCTGTCTACCTTCACTGAATACATCAA
 GAACATCATCAATACCTCCATCCTGAACCTGCGCTACGAATCCAATCACC
 TGATCGACCTGTCTCGCTACGCTTCCAAAATCAACATCGGTTCTAACTT
 AACTTCGATCCGATCGACAAGAATCAGATCCAGCTGTTCAATCTGGAAT
 5 CTTCCAAAATCGAAGTTATCCTGAAGAATGCTATCGTATACAACCTCTATG
 TACGAAAACCTTCTCCACCTCCTTCTGGATCCGTATCCCAAATACTTCAAC
 TCCATCTCTCTGAACAATGAATACACCATCATCAACTGCATGGAAAACA
 ATTCTGGTTGGAAAGTATCTCTGAACTACGGTGAAATCATCTGGACTCTG
 CAGGACACTCAGGAAATCAAACAGCGTGTTGTATTCAAATACTCTCAGA
 10 TGATCAACATCTCTGACTACATCAATCGCTGGATCTTCGTTACCATCACC
 AACAATCGTCTGAATAACTCCAAAATCTACATCAACGCCGTCTGATCGA
 CCAGAAACCGATCTCCAATCTGGGTAACATCCACGTTCTAATAACATCAT
 GTTCAAACCTGGACGGTTGTCGTGACACTACCGCTACATCTGGATCAAAT
 ACTTCAATCTGTTGACAAAGAACTGAACGAAAAAGAAATCAAAGACCT
 15 GTACGACAACCAGTCCAATTCTGGTATCCTGAAAGACTTCTGGGGTGACT
 ACCTGCAGTACGACAAACCGTACTACATGCTGAATCTGTACGATCCGAA
 CAAATACGTTGACGTCAACAATGTAGGTATCCGCGGTTACATGTACCTG
 AAAGGTCCGCGTGGTTCTGTTATGACTACCAACATCTACCTGAACTCTTC
 CCTGTACCGTGGTACCAAATTCATCATCAAGAAATACGCGTCTGGTAAC
 20 AAGGACAATATDCGCAACAATGATCGTGTATACATCAATGTTGTAGTTA
 AGAACAAAGAATACCGTCTGGCTACCAATGCTTCTCAGGCTGGTGTAGA
 AAAGATCTTGTCTGCTCTGGAAATCCCGGACGTTGGTAATCTGTCTCAGG
 TAGTTGTAATGAAATCCAAGAACGACCAGGGTATCACTAACAATGCAA
 AATGAATCTGCAGGACAACAATGGTAACGATATCGGTTTCATCGGTTTCC
 25 ACCAGTTCAACAATATCGCTAAACTGGTTGCTTCCAACTGGTACAATCGT
 CAGATCGAACGTTCTCTCGCACTCTGGGTTGCTCTTGGGAGTTCATCCC
 GGTTGATGACGGTTGGGGTGAACGTCCGCTGTAACCCGGGAAAGCTT

This gene has been synthesized using a large number of oligomers of
 approximately 60-65 bases corresponding to the sequences of the + and - strands.
 30 The oligomers had overlaps of 7 bases. The oligomers were allowed to anneal and
 were ligated to form 5 subunits of 250-300 base pairs each. Each subunit had been
 designed to have restriction sites at their termini which allowed them to be

assembled in the right order to form the complete gene. On confirmation there was shown that the correct gene had 7 deletion errors. These errors were repaired *using in vitro* mutagenesis and the repair sites sequenced to confirm.

Example 8. Synthesis and cloning of a synthetic gene encoding

5 rBoNTB (H_C)

The C fragment for botulism toxin serotype B of Whelan was studied and the portion of the protein having the sequence

FNKYNSEILNNIILNRLRYKDNNLIDLSGYGAKVEVYDGVELNDKNQF
 KLTSSANSKIRVTQNQNIIFNSVFLDFSVSFWIRIPKYKNDGIQNYIHNEYTIIN
 10 CMKNNSGWKISIRGNRITWTLIDINGKTKSVFFEYNIREDISEYINRWFFVTIT
 NNLNNAKIYINGKLESNTDIKDIREVIANGEIIFKLDGDIDRTQFTWMKYFSIF
 NTELSQSNIERYKIQSYSEYLKDFWGNPLMYNKEYYMFNAGNKNSYIKLK
 KDSPVGEILTRSKYNQNSKYINYRDLYIGEKFIRRKSNSSQINDDIVRKEDI
 YLDF-NLNQEQRVYTYKKFKKEEEKLFLAPISDSDEFYNTIQIKEYDEQPTY
 15 SCQLLFKKDEESTDEIGLIGIHRFYESGIVFEEYKDYFCISKWYLKEVKRKP
 YNLKLGCNWQFIPKDEGWTE

was defined as the C fragment.

The synthetic gene for expression in *E. coli* was produced in the manner described for synthesis of the gene for the C fragment of the A strand, namely, using
 20 a large number of oligomers of approximately 60-65 bases corresponding to the sequences of the ₊ and ₋ strands with overlaps of 7 bases. The oligomers were allowed to anneal and were ligated to form subunits of 250-300 base pairs each. Each subunit had been designed to have restriction sites at their termini which allowed them to be assembled in the right order to form the complete gene. the
 25 synthetic gene for encoding the c fragment of the B toxin was as follows:

ATGGCTTTCAACAAATACAATTCGAAATCCTGAACAATATCATC
 CTGAACCTGCGTTACAAAGACAACAATCTGATCGATCTGTCTGGTTACGG
 TGCTAAAGTTGAAGTATACGACGGTGTTGAACTGAATGACAAGAACCAG
 TTCAAACCTGACCTCTTCCGCTAACTCTAAGATCCGTGTTACTCAGAATCA
 30 GAACATCATCTTCAACTCCGTATTCCTGGACTTCTCTGTTTCCTTCTGGAT
 CCGTATCCCGAAATACAAGAACGACGGTATCCAGAATTACATCCACAAT
 GAATACACCATCATCAACTGCATGAAGAATAACTCTGGTTGGAAGATCT

CCATCCGCGGTAACCGTATCATCTGGACTCTGATCGATATCAACGGTAAG
 ACCAAATCTGTATTCTTCGAATACAACATCCGTGAAGACATCTCTGAATA
 CATCAATCGCTGGTTCTTCGTTACCATCACCAATAACCTGAACAATGCTA
 AAATCTACATCAACGGTAAACTGGAATCTAATAACCGACATCAAAGACAT
 5 CCGTGAAGTTATCGCTAACGGTGAAATCATCTTCAAACCTGGACGGTGAC
 ATCGATCGTACCCAGTTCATCTGGATGAAATACTTCTCCATCTTCAACAC
 CGAACTGTCTCAGTCCAATATCGAAGAACGGTACAAGATCCAGTCTTAC
 TCCGAATACCTGAAAGACTTCTGGGGTAATCCGCTGATGTACAACAAAG
 AATACTATATGTTCAATGCTGGTAACAAGAAGTCTTACATCAAACCTGAA
 10 GAAAGACTCTCCGGTTGGTGAATCCTGACTCGTTCCAAATACAACCAGA
 ACTCTAAATACATCAACTACCGCGACCTGTACATCGGTGAAAAGTTCATC
 ATCCGTCGCAAATCTAACTCTCAGTCCATCAATGAGACATCGTACGTAAA
 GAAGACTACATCTACCTGGACTTCTTCAACCTGAATCAGAATGGCGTGTA
 TACACCTACAAGTACTTCAAGAAAGAAGAAGAAAAGCTTTTCTGGCTC
 15 CGATCTCTGATTCCGACGAACTCTACAACACCATCCAGATCAAAGAATA
 CGACGAACAGCCGACCTACTCTTGCCAGCTGCTGTTCAAGAAAGATGAA
 GAATCTACTGACGAAATCGGTCTGATCGGTATCCACCGTTTCTACGAATC
 TGGTATCGTATTCGAAGAATACAAAGACTCTTCTGCATCTCCAAATGGTA
 CCTGAAGGAAGTTAAACGCAAACCGTACAACCTGAAACTGGGTTGCAAT
 20 TGGCAGTTCATCCCGAAAGACGAAGGTTGGACCGAATAGTAACCTCTAG
 AGTCGAGGCCTGCAG

Cloning:

Supernatants of sonicated, IPTG-induced recombinant pMAL fusion *E. coli*
 cultures were tested for the presence of the botulinum H_C expression product by
 25 ELISA and SDS-PAGE gels stained with coomassie brilliant blue were
 unsuccessful. Attempts to express H_C fragment as a non-fusion product were
 unsuccessful. Initial characterization of plasmid DNA from putative clones in
 pKK233-2 demonstrated an insert of the expected size was present. In addition,
 SDS-PAGE indicated the presence of a protein of approximately 50 kDa after
 30 induction. However, the recombinants appeared unstable and further preparations of
 this and other cultures failed to reproduce these results. This approach was
 subsequently abandoned in favor of the fusion product expression.

Example 9 Immunization Trials:

Although attempts to quantitate expressed H_C fusion products were unsuccessful, limited immunization trials were performed on mice to evaluate the vaccine potential of the product. Initial vaccination employed concentrated, crude
5 *E. coli* lysate with complete Freund's adjuvant. Two weeks later, animals were boosted with amylose column-purified expression product with Freund's incomplete adjuvant. At this time, a second group of five animals received amylose purified product in Freund's incomplete adjuvant as a single vaccination. After two additional weeks, both groups were challenged intraperitoneally with a dose of 3
10 LD₅₀ of toxin. All eleven animals receiving two immunizations with H_C survived while six of the twelve control animals receiving pMAL vector alone died. Likewise, all five animals receiving one H_C vaccination survived while animals receiving the pMAL vector alone died.

Four weeks after the initial challenge with 3 LD₅₀ of toxin, nine of the eleven
15 animals who had received two immunizations were exposed to 30, 300, or 1200 LD₅₀ doses of toxin. The animals succumbing to the toxin challenge of 30 and 300 LD₅₀ did not exhibit fatality typical of botulinum toxin poisoning in that they appeared healthy after 18 hours, but were dead a few hours thereafter. In contrast, the animal which died from the 1200 LD₅₀ dose appeared moribund when examined
20 at 18 hours and remained so until death. This reaction is consistent with symptoms usually observed with botulinum toxin-induced paralysis. Additional data on second challenge is shown on Table 5. Hence, it was shown that immunization with the genetically engineered toxin protected against large doses of the toxin.

It is also possible to produce antibodies using the genetically
25 engineered toxin. Because the toxin is not disease-producing in the animal, it is possible to produce large amounts of antitoxin more cheaply. It is also possible to produce antitoxin using hybridoma technology.

TABLE 5

**PROTECTION OF MICE IMMUNIZED WITH H_C OF A TOXIN
DERIVED FROM SYNTHETIC GENE (# of deaths/total animals)**

Calculated challenge dose (LD ₅₀)	Control (vector without insert)	Protected (Vector with insert)
4	2/3	0/3
10		0/3
30	3/4	0/3
100		0/3
300		0/3
1000		0/3

3000 0/1

- 5 The animals received vaccinations of crude lysated cell material at 0, 2 and 4 weeks. Challenges were administered intraperitoneally with serotype A toxin at 5 weeks.

Example 10 rBoNTA(H_C) purification and protective effect

- Recombinant BoNTA(H_C) peptide was produced recombinantly in yeast. The first
- 10 step in the purification process for BoNTA(H_C) was a Streamline expanded bed chromatography column. The product was eluted by a sodium chloride step gradient. Product eluted from the expanded bed chromatography column was estimated to be 10% pure with a total protein concentration of 0.92 mg/ml. After dialyzing the salt away, the material was loaded onto a mono S cation exchange column for further purification.
- 15 Western blot and ELISA data indicated that BoNTA(H_C) eluted from the column at 110 mM sodium chloride. The Mono S pool was subjected to HIC as a final purification step and thus, the material was adjusted to 1.5 M ammonium sulfate. The Mono S product was loaded onto a HIC column and eluted with a gradient of decreasing ammonium sulfate. Product eluted at 1.04 M ammonium sulfate and BoNTA(H_C) immunologically positive
- 20 fractions were combined and dialyzed to remove ammonium sulfate. Only a 50 kDa BoNTA(H_C) band was detected by SDS-PAGE and Western blot analysis and was judged to be greater than 95% pure after the final step. Protective effect of this purified material was measured by immunizing mice with 1 dose followed by challenge with 1000 LD₅₀ of BoNTA(H_C). The results are shown in Table 6 below.

Table 6 Potency assay: 1 dose followed by challenge with 1000 LD50 of BoNTA(H_C)

Dose (μg)	survival
10	10/10
2.5	10/10
0.625	10/10
0.156	7/10
0.039	2/10
0.0098	0/10
0.0024	0/10

Example 11 rBoNTB(H_C) purification and protective effect

5 Recombinant BoNTC(H_C) peptide was produced recombinantly in yeast. The first separation technique employed for the purification process for BoNTB(H_C) was Streamline chromatography (Pharmacia), which is a single pass expanded bed adsorption operation where proteins can be recovered from crude feed stock or cell lysate without prior clarification. Significant clean-up was accomplished in this step as the MES buffer

10 system prohibited binding of a large percentage of unwanted proteins to the SP resin. Protein was loaded onto the column at a concentration of 123 mg/mL-resin, using 20 mM MES buffer, pH 5.7 with 10 mM NaCl. The product pool was eluted in a single step. Under the conditions investigated, on average 3.9% of the total protein loaded was recovered in the elution peak, and the product pool was approximately 70% BoNTB(H_C)

15 fragment based on SDS-PAGE.

The second chromatography step in the process utilizes Poros HS, another strong cation exchange resin. The buffer system was similar to that used for Streamline SP, however enhanced selectivity of Poros HS enriched the product peak to about 85% purity. The product peak eluted during the gradient at approximately 130 mM NaCl. Strongly

20 bound proteins were eluted with 1 M NaCl.

The final chromatography step utilized a Poros PI column. Analysis of the PI fractions by SDS-PAGE and IEF revealed that the product band, a single band at 50 kD

on SDS-PAGE, was present in the pH 8.0 fraction. Analysis of purified BoNTB(H_C) fragment by 2-D electrophoresis resulted in one major spot and two minor, faint spots from the PI-peak 1 fraction. Peak 2 contained several spots at two different molecular weights corresponding to 50 kD and 47 kD. Presumably these spots represent different isoforms. IEF banding patterns detected in the first dimension are in agreement with those seen in Phast IEF for the two peaks. The protective efficacy of this material was determined by potency assay of 1 dose followed by challenge with 1000 LD₅₀ of BoNTB(H_C). The results are shown in the following Table 7.

Table 7 BoNTB(H_C)

Dose (μg)	survival
10	10/10
2.5	10/10
0.625	10/10
0.156	6/10
0.039	1/10
0.0098	0/10
0.0024	0/10

10

Example 12 rBoNTC(H_C) purification and protective effect

Recombinant BoNTC(H_C) peptide was produced recombinantly in yeast. The initial chromatography step used for the purification process for BoNTC₁(H_C) was a Mono Q anion-exchange column. The column was equilibrated with 50 mM sodium phosphate, 0.2% (W/V) CHAPS, 2 mM EDTA, pH 7.0. The CHAPS was incorporated into the column buffers to allow product to elute from the column over a narrower sodium chloride concentration. Fractions positive for BoNTC₁(H_C) by Western analysis were pooled and adjusted to 1 M ammonium sulfate. A moderate precipitate formed which was removed by passing the material through a 0.2 μ filtration unit. The clarified Mono Q product pool was subjected to hydrophobic interaction chromatography using a Pharmacia alkyl superose column. This final step removed the remainder of the impurities liberating BoNTC₁(H_C) product which was estimated to be greater than 98% pure as judged by SDS/PAGE. Protective effect of this purified material was measured by immunizing mice

20

with 1 dose followed by challenge with 1000 LD50 of BoNTC₁(H_C). The results are shown in Table 8 below.

Table 8. Potency Assay: One dose followed by challenge with 1000 LD50 of BoNTC₁(H_C)

Dose (μg)	Survival
8.1	10/10
2.7	10/10
0.9	10/10
0.3	9/10
0.1	4/10
0.033	0/10
0.011	0/10

5

For purposes of clarity of understanding, the foregoing invention has been described in some detail by way of illustration and example in conjunction with specific embodiments, although other aspects, advantages and modifications will be apparent to those skilled in the art to which the invention pertains. The foregoing description and examples are intended to illustrate, but not limit the scope of the invention. Modifications of the above-described modes for carrying out the invention that are apparent to persons of skill in medicine, immunology, hybridoma technology, pharmacology, and/or related fields are intended to be within the scope of the invention, which is limited only by the appended claims.

All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

From : Pichia AOX1 gene REFORMAT of: aox1struc.dat check: 7029 from: 1 to: 1992 27-MAY-1987 11:40				AAAcid	Codon	Number	Fraction
From : Pichia AOX2 gene				Met	ATG	90.00	1.00
REFORMAT of: aox2struc.dat check: 9841				Ile	ATA	0.00	0.00
from: 1 to: 1992 27-MAY-1987 11:41				Ile	ATT	91.00	0.56
From : Pichia DAS1 gene				Ile	ATC	72.00	0.44
REFORMAT of: das1struc.dat check: 3191				Thr	ACG	5.00	0.03
from: 1 to: 2124 22-APR-1987 15:08				Thr	ACA	8.00	0.03
From : Pichia DAS2 gene				Thr	ACT	86.00	0.50
REFORMAT of: das2struc.dat check: 5479				Thr	ACC	74.00	0.43
from: 1 to: 2124 15-JUN-1987 14:33				Trp	TGG	39.00	1.00
From : Pichia GAP gene				End	TGA	0.00	0.00
REFORMAT of: gapstruc.dat check: 9059				Cys	TGT	35.00	0.83
from: 1 to: 1002 15-JUN-1987 14:38				Cys	TGC	7.00	0.17
AAAcid Codon Number Fraction				End	TAG	1.00	0.20
				End	TAA	4.00	0.80
				Tyr	TAT	18.00	0.12
				Tyr	TAC	128.00	0.88
				Leu	TTG	120.00	0.52
				Leu	TTA	21.00	0.08
				Phe	TTT	24.00	0.19
				Phe	TTC	104.00	0.81
				Ser	TCG	8.00	0.03
				Ser	TCA	14.00	0.07
				Ser	TCT	89.00	0.47
				Ser	TCC	71.00	0.37
				Arg	CGG	2.00	0.01
				Arg	CGA	0.00	0.00
				Arg	CGT	26.00	0.18
				Arg	CGC	0.00	0.00
				Gln	CAG	31.00	0.34
				Gln	CAA	59.00	0.66
				His	CAT	11.00	0.13
				His	CAC	77.00	0.88
				Leu	CTG	35.00	0.15
				Leu	CTA	7.00	0.03
				Leu	CTT	43.00	0.18
				Leu	CTC	7.00	0.03
				Pro	CCG	0.00	0.00
				Pro	CCA	97.00	0.57
				Pro	CCT	68.00	0.39
				Pro	CCC	7.00	0.04

Table 1
PICHIA CODON USAGE TABLE

CLAIMS:

1. A nucleic acid encoding the carboxy-terminal portion of the heavy chain (H_C) of botulinum neurotoxin (BoNT) selected from the group consisting of BoNT serotype A, BoNT serotype B, BoNT serotype C₁, BoNT serotype D, BoNT serotype E, BoNT serotype F, and BoNT serotype G, wherein said nucleic acid is expressable in a recombinant organism selected from *Escherichia coli* and *Pichia pastoris*.
5
2. The nucleic acid of claim 1, wherein said nucleic acid comprises a nucleic acid sequence selected from SEQ ID No:1 (serotype A), SEQ ID No:7 (serotype B), SEQ ID No:9 (serotype C₁), SEQ ID No:11 (serotype D), SEQ ID No:13 (serotype E), SEQ ID No:15 (serotype F), and SEQ ID No:17 (serotype G).
10
3. The nucleic acid of claim 1, wherein the nucleic acid encodes an H_C amino acid sequence of BoNT selected from SEQ ID No:2 (serotype A), SEQ ID No:8 (serotype B), SEQ ID No:10 (serotype C₁), SEQ ID No:12 (serotype D), SEQ ID No:14 (serotype E), SEQ ID No:16 (serotype F), and SEQ ID No:18 (serotype G).
15
4. A nucleic acid encoding the amino-terminal portion of the heavy chain (H_N) of botulinum neurotoxin (BoNT) selected from the group consisting of BoNT serotype B, BoNT serotype C₁, BoNT serotype D, BoNT serotype E, BoNT serotype F, and BoNT serotype G, wherein said nucleic acid is expressable in a recombinant organism selected from *Escherichia coli* and *Pichia pastoris*.
20
5. The nucleic acid of claim 4, wherein said nucleic acid comprises a nucleic acid sequence selected from SEQ ID No:21 (serotype B), SEQ ID No:23 (serotype C₁), SEQ ID No:25 (serotype D), SEQ ID No:27 (serotype E), SEQ ID No:29 (serotype F), and SEQ ID No:31 (serotype G).
25
6. The nucleic acid of claim 4, wherein the nucleic acid encodes an H_N amino acid sequence of BoNT selected from SEQ ID No:22 (serotype B), SEQ ID No:24 (serotype C₁), SEQ ID No:26 (serotype D), SEQ ID No:28 (serotype E), SEQ ID No:30 (serotype F), and SEQ ID No:32 (serotype G).
30

7. The nucleic acid of any one of claims 1, 3, 4, or 6, wherein the sequence of the nucleic acid is designed by selecting at least a portion of the codons encoding H_C from codons preferred for expression in a host organism.
- 5
8. The nucleic acid of claim 7, wherein the host organism is selected from gram negative bacteria, yeast, and mammalian cell lines.
9. The nucleic acid of claim 8, wherein the host organism is *Escherichia coli* or
- 10 *Pichia pastoris*.
10. The nucleic acid of any one of claims 1, 3, 4, or 6, wherein the nucleic acid sequence encoding H_C is designed by selecting codons encoding H_C which codons provide H_C sequence enriched in guanosine and cytosine residues.
- 15
11. The nucleic acid of any one of claims 1, 3, 4, or 6, wherein said nucleic acid is a synthetic nucleic acid.
12. The nucleic acid of any one of claims 1, 3, 4, or 6, wherein said nucleic acid
- 20 encoding H_C or H_N is expressed in a recombinant host organism with higher yield than a second nucleic acid fragment encoding the same H_C sequence, said second nucleic acid fragment having the wild-type *Clostridium botulinum* sequence of H_C.
13. An expression vector comprising the nucleic acid of any one of claims 1, 3,
- 25 4, or 6, whereby H_C or H_N is expressed upon transfection of a host organism with said expression vector.
14. A method of preparing a polypeptide comprising the carboxy-terminal portion of the heavy chain (H_C) of botulinum neurotoxin (BoNT) or the amino-
- 30 terminal portion of the heavy chain (H_N) of botulinum neurotoxin (BoNT) selected from the group consisting of BoNT serotype A, BoNT serotype B, BoNT serotype C, BoNT serotype D, BoNT serotype E, BoNT serotype F, and BoNT serotype G,

said method comprising culturing a recombinant host organism transfected with the expression vector of claim 13 under conditions wherein H_C or H_N is expressed.

15 15. The method of claim 14, wherein the recombinant host organism is a eukaryote.

16. The method of claim 14, further comprising recovering insoluble protein from said host organism, whereby a fraction enriched in H_C or H_N is obtained.

10 17. The method of claim 16, wherein said host organism is *Pichia pastoris*.

18. An immunogenic composition comprising the carboxy-terminal portion of the heavy chain (H_C) of botulinum neurotoxin (BoNT) selected from the group consisting of BoNT serotype A, BoNT serotype B, BoNT serotype C, BoNT
15 serotype D, BoNT serotype E, BoNT serotype F, and BoNT serotype G.

19. The immunogenic composition of claim 18, wherein H_C is prepared by culturing a recombinant organism transfected with an expression vector encoding H_C.

20

20. The immunogenic composition of claim 19, wherein an insoluble protein fraction enriched in H_C is recovered from said recombinant organism.

21. An immunogenic composition comprising the amino-terminal portion of the
25 heavy chain (H_N) of botulinum neurotoxin (BoNT) selected from the group consisting of BoNT serotype A, BoNT serotype B, BoNT serotype C, BoNT serotype D, BoNT serotype E, BoNT serotype F, and BoNT serotype G.

22. The immunogenic composition of claim 15, wherein H_N is prepared by
30 culturing a recombinant organism transfected with an expression vector encoding H_N.

23. The immunogenic composition of claim 16, wherein an insoluble protein fraction enriched in H_N is recovered from said recombinant organism.

24. An immunogenic composition comprising a polypeptide comprising epitopes
5 contained in the carboxy-terminal portion of the heavy chain (H_C) of botulinum neurotoxin (BoNT) or the amino-terminal portion of the heavy chain (H_N) of botulinum neurotoxin (BoNT) selected from the group consisting of BoNT serotype A, BoNT serotype B, BoNT serotype C, BoNT serotype D, BoNT serotype E, BoNT serotype F, and BoNT serotype G, said epitopes eliciting protective immunity
10 toward the respective BoNT serotype.

25. The immunogenic composition of claim 25, wherein said immunogenic composition elicits an ELISA response to the respective BoNT serotype in an animal, said ELISA response being detectable upon 100-fold dilution of serum from
15 said animal.

FIGURE 1

A: BoNTA(H_c) synthetic gene (Version #1) SEQ ID NO: 1

GAATTCGAAACGatgCGTCTGCTGTCTACCTTCACTGAATACATCAAGAAC
 ATCATCAATACCTCCATCCTGAACCTGCGCTACGAATCCAATCACCTGAT
 CGACCTGTCTCGCTACGCTTCCAAAATCAACATCGGTTCTAAAGTTAACT
 TCGATCCGATCGACAAGAATCAGATCCAGCTGTTCAATCTGGAATCTTCC
 AAAATCGAAGTTATCCTGAAGAATGCTATCGTATACAACCTCTATGTACG
 AAAACTTCTCCACCTCCTTCTGGATCCGTATCCCGAAATACTTCAACTCC
 ATCTCTCTGAACAATGAATACACCATCATCAACTGCATGGAAAACAATT
 CTGGTTGGAAAGTATCTCTGAACTACGGTGAAATCATCTGGACTCTGCAG
 GAACTCAGGAAATCAAACAGCGTGTTGTATTCAAATACTCTCAGATGA
 TCAACATCTCTGACTACATCAATCGCTGGATCTTCGTTACCATCACCAC
 AATCGTCTGAATAACTCCAAAATCTACATCAACGGCCGTCTGATCGACC
 AGAAACCGATCTCCAATCTGGGTAAACATCCACGCTTCTAATAACATCATG
 TTCAAACCTGGACGGTTGTCTGACACTCACCGCTACATCTGGATCAAATA
 CTTCAATCTGTTTCGACAAAGAACTGAACGAAAAAGAAATCAAAGACCTG
 TACGACAACCAAGTCCAATTCTGGTATCCTGAAAGACTTCTGGGGTGACTA
 CCTGCAGTACGACAAACCGTACTACATGCTGAATCTGTACGATCCGAAC
 AAATACGTTGACGTCAACAATGTAGGTATCCGCGGTTACATGTACCTGA
 AAGGTCCGCGTGGTTCTGTTATGACTACCAACATCTACCTGAACTCTTCC
 CTGTACCGTGGTACCAAATTCATCATCAAGAAATACGCGTCTGGTAACA
 AGGACAATATCGTTTCGCAACAATGATCGTGTATACATCAATGTTGTAGTT
 AAGAACAAGAATAACCGTCTGGCTACCAATGCTTCTCAGGCTGGTGTAG
 AAAAGATCTTGTCTGCTCTGGAATCCCGGACGTTGGTAATCTGTCTCAG
 GTAGTTGTAATGAAATCCAAGAACGACCAGGGTATCACTAACAATGCA
 AAATGAATCTGCAGGACAACAATGGTAACGATATCGGTTTCATCGGTTT
 CCACCAGTTCAACAATATCGCTAACTGGTTGCTTCCAACCTGGTACAATC
 GTCAGATCGAACGTTCTCTCGCACTCTGGGTTGCTCTTGGGAGTTCATC
 CCGGTTGATGACGGTTGGGGTGAACGTCCGCTGtaaGAATTC

B: BoNTA(H_c) encoded protein (Version #1) SEQ ID NO: 2

MRLLSTFTEYIKNIINTSILNLRYESNHLIDLSRYASKINIGSKVNFDPIDKNQI
 QLFNLESSKIEVILKNAIVYNSMYENFSTSWIRIPKYFNSISLNNEYTIINCME
 NNSGWKVSILNYGEIHWTLQDTQEIKQRVVFKYSQMINISDYINRWIFVTITNN
 RLNNSKIYINGRLIDQKPISNLGNIHASNNIMFKLDGCRDTHR YIWIKYFNLF
 DKELNEKEIKDL YDNQSN SGILKDFWGDYLQYDKPY YMLNLYDPNKYVDV
 NNVGIRGYMYLKGPRGSVMTTNIYLNSSLYRGTKFIKKYASGNKDNIVRN
 NDRVYINVVVKNKEYRLATNASQAGVEKILSALEIPDVGNLSQVVVMKSKN
 DQGITNKCKMNLQDNNGNDIGFIGFHQFNNI AKLVASNWYNRQIERSSRTL
 GCSWEFIPVDDGWGERPL*

FIGURE 2**A: BoNTA(H_c) synthetic gene (version #2) SEQ ID NO: 3**

GAATTCGAAACGatgTCTACCTTCACTGAATACATCAAGAACATCATCAAT
 ACCTCCATCCTGAACCTGCGCTACGAATCCAATCACCTGATCGACCTGTC
 TCGCTACGCTTCCAAAATCAACATCGGTTCTAAAGTTAACTTCGATCCGA
 TCGACAAGAATCAGATCCAGCTGTTCAATCTGGAATCTTCCAAAATCGA
 AGTTATCCTGAAGAATGCTATCGTATACAACTCTATGTACGAAAACCTTCT
 CCACCTCCTTCTGGATCCGTATCCCGAAATACTTCAACTCCATCTCTCTG
 AACAAATGAATACACCATCATCAACTGCATGGAAAACAATTCTGGTTGGA
 AAGTATCTCTGAACTACGGTGAAATCATCTGGACTCTGCAGGACACTCA
 GGAAATCAAACAGCGTGTTGTATTCAAATACTCTCAGATGATCAACATCT
 CTGACTACATCAATCGCTGGATCTTCGTTACCATCACCAACAATCGTCTG
 AATAACTCCAAAATCTACATCAACGGCCGTCTGATCGACCAGAAACCGA
 TCTCCAATCTGGGTAACATCCACGCTTCTAATAACATCATGTTCAAACCTG
 GACGGTTGTCGTGACACTCACCGCTACATCTGGATCAAATACTTCAATCT
 GTTCGACAAAGAACTGAACGAAAAAGAAATCAAAGACCTGTACGACAA
 CCAGTCCAATTCTGGTATCCTGAAAGACTTCTGGGGTGACTACCTGCAGT
 ACGACAAACCGTACTACATGCTGAATCTGTACGATCCGAACAAATACGT
 TGACGTCAACAATGTAGGTATCCGCGGTTACATGTACCTGAAAGGTCCG
 CGTGGTTCTGTTATGACTACCAACATCTACCTGAACTCTTCCCTGTACCG
 TGGTACCAAATTCATCATCAAGAAATACGCGTCTGGTAACAAGGACAAT
 ATCGTTCGCAACAATGATCGTGTATACATCAATGTTGTAGTTAAGAACAA
 AGAATACCGTCTGGCTACCAATGCTTCTCAGGCTGGTGTAGAAAAGATC
 TTGTCTGCTCTGGAAATCCCGGACGTTGGTAATCTGTCTCAGGTAGTTGT
 AATGAAATCCAAGAACGACCAGGGTATCACTAACAATGCAAAATGAAT
 CTGCAGGACAACAATGGTAACGATATCGGTTTCATCGGTTTCCACCAGTT
 CAACAATATCGCTAACTGGTTGCTTCCAACTGGTACAATCGTCAGATCG
 AACGTTCTCTCGCACTCTGGGTTGCTCTTGGGAGTTCATCCCGGTTGAT
 GACGGTTGGGGTGAACGTCCGCTGtaaGAATTC

B: BoNTA(H_c) encoded protein (Version #2) SEQ ID NO: 4

MSTFTEYIKNIINTSILNLRYESNHLIDLSRYASKINIGSKVNFDPIDKNQIQLF
 NLESSKIEVILKNAIVYNSMYENFSTSFWIRIPKYFNSISLNNEYTIINCMENNS
 GWKVS LNYGEI IWLQDTQEIKQRVVF KY SQMINISDYINRWIFVTITNNRLN
 NSKIYINGRLIDQKPISNLGNIHASNNIMFKLDGCRDTHR YIWIKYFN LFDKE
 LNEKEIKDL YDNQSN SGILKDFWGDY LQYDKPY YMLNLYDPNKYVDVNNV
 GIRGYMYLKGPRGSVM TTN IYLNSSLYRGT KFIKKYASGNKDNI VRNNDRV
 YINVVVK NKEYRLATNASQAGVEKILSALEIPDVGNLSQVVVMKSKNDQGI
 TNKCKMNLQDNNGNDIGFIGFHQFN NIAKLVASNWYNRQIERSSRTLGC SW
 EFIPVDDGWGERPL*

FIGURE 3**A: BoNTA(H_c) synthetic gene (Version #3) SEQ ID NO: 5**

GAATTCGAAACGatgGCCTCTACCTTCACTGAATACATCAAGAACATCATC
AATACCTCCATCCTGAACCTGCGCTACGAATCCAATCACCTGATCGACCT
GTCTCGCTACGCTTCCAAAATCAACATCGGTTCTAAAGTTAACTTCGATC
CGATCGACAAGAATCAGATCCAGCTGTTCAATCTGGAATCTTCCAAAAT
CGAAGTTATCCTGAAGAATGCTATCGTATACAACCTCTATGTACGAAAAC
TCTCCACCTCCTTCTGGATCCGTATCCCGAAATACTTCAACTCCATCTCTC
TGAACAATGAATACACCATCATCAACTGCATGGAAAACAATTCTGGTTG
GAAAGTATCTCTGAACTACGGTGAAATCATCTGGACTCTGCAGGACACT
CAGGAAATCAAACAGCGTGTTGTATTCAAATACTCTCAGATGATCAACA
TCTCTGACTACATCAATCGCTGGATCTTCGTTACCATCACCAACAATCGT
CTGAATAACTCCAAAATCTACATCAACGGCCGTCTGATCGACCAGAAAC
CGATCTCCAATCTGGGTAACATCCACGCTTCTAATAACATCATGTTCAA
CTGGACGGTTGTCGTGACACTCACCGCTACATCTGGATCAAATACTTCAA
TCTGTTTCGACAAAGAACTGAACGAAAAAGAAATCAAAGACCTGTACGAC
AACCAGTCCAATTCTGGTATCCTGAAAGACTTCTGGGGTGACTACCTGCA
GTACGACAAACCGTACTACATGCTGAATCTGTACGATCCGAACAAATAC
GTTGACGTCAACAATGTAGGTATCCGCGGTTACATGTACCTGAAAGGTC
CGCGTGGTTCTGTTATGACTACCAACATCTACCTGAACTCTTCCCTGTAC
CGTGGTACCAAATTCATCATCAAGAAATACGCGTCTGGTAACAAGGACA
ATATCGTTTCGCAACAATGATCGTGTATACATCAATGTTGTAGTTAAGAAC
AAAGAATACCGTCTGGCTACCAATGCTTCTCAGGCTGGTGTAGAAAAGA
TCTTGTCTGCTCTGGAAATCCCGGACGTTGGTAATCTGTCTCAGGTAGTT
GTAATGAAATCCAAGAACGACCAGGGTATCACTAACAATGCAAAATGA
ATCTGCAGGACAACAATGGTAACGATATCGGTTTCATCGGTTTCCACCAG
TTCAACAATATCGCTAAACTGGTTGCTTCCAACCTGGTACAATCGTCAGAT
CGAACGTTCTCTCGCACTCTGGGTTGCTCTTGGGAGTTCATCCCGGTTG
ATGACGGTTGGGGTGAACGTCCGCTGtaaGAATTC

B: BoNTA(H_c) encoded protein (Version #3) SEQ ID NO: 6

MASTFTEYIKNIINTSILNLRYESNHLIDLSRYASKINIGSKVNFDPIDKNQIQL
FNLESSKIEVILKNAIVYNSMYENFSTSFWIRIPKYFNSISLNNEYTIINCMENN
SGWKVSLNYGEIWTLQDTQEIQRVVFYKYSQMINISDYINRWIFVTITNRL
NNSKIYINGRLIDQKPISNLGNIHASNNIMFKLDGCRDTHRYTWIKYFNLFDK
ELNEKEIKDL YDNQSNL KDFWGDY LQYDKPYMNLNLYDPNKYVDVNN
VGIRGYMYLKGRGSVMTTNIYLNSSLYRGTKFIKKYASGNKDNIVRNDR
VYINVVVKNKEYRLATNASQAGVEKILSALEIPDVGNLSQVVVMKSKNDQ
GITNKCKMNLQDNNNGNDIGFIGFHQFNNAIKLVASNWYNRQIERSSRTLGC
SEFIPVDDGWGERPL*

FIGURE 4

A: BoNTB(H_c) synthetic gene SEQ ID NO: 7

GAATTCACGatgGCCAACAAATACAATTCGAAATCCTGAACAATATCAT
 CCTGAACCTGCGTTACAAAGACAACAATCTGATCGATCTGTCTGGTTACG
 GTGCTAAAGTTGAAGTATACGACGGTGTGAACTGAATGACAAGAACCA
 GTTCAAACCTGACCTCTTCCGCTAACTCTAAGATCCGTGTTACTCAGAATC
 AGAACATCATCTTCAACTCCGTATTCCTGGACTTCTCTGTTTCCTTCTGGA
 TTCGTATCCCGAAATACAAGAACGACGGTATCCAGAATTACATCCACAA
 TGAATACACCATCATCAACTGCATGAAGAATAACTCTGGTTGGAAGATC
 TCCATCCGCGGTAACCGTATCATCTGGACTCTGATCGATATCAACGGTAA
 GACCAAATCTGTATTCTTCGAATACAACATCCGTGAAGACATCTCTGAAT
 ACATCAATCGCTGGTTCTTCGTTACCATCACCAATAACCTGAACAATGCT
 AAAATCTACATCAACGGTAACTGGAATCTAATACCGACATCAAAGACA
 TCCGTGAAGTTATCGCTAACGGTGAAATCATCTTCAAACCTGGACGGTGA
 CATCGATCGTACCCAGTTCATCTGGATGAAATACTTCTCCATCTTCAACA
 CCGAACTGTCTCAGTCCAATATCGAAGAACGGTACAAGATCCAGTCTTA
 CTCCGAATACCTGAAAGACTTCTGGGGTAATCCGCTGATGTACAACAAA
 GAATACTATATGTTCAATGCTGGTAACAAGAACTCTTACATCAAACCTGA
 AGAAAGACTCTCCGGTTGGTGAAATCCTGACTCGTTCCAAATACAACCA
 GAACTCTAAATACATCAACTACCGCGACCTGTACATCGGTGAAAAGTTC
 ATCATCCGTCGCAAATCTAACTCTCAGTCCATCAATGATGACATCGTACG
 TAAAGAAGACTACATCTACCTGGACTTCTTCAACCTGAATCAGGAATGG
 CGTGTATACACCTACAAGTACTTCAAGAAAGAAGAAGAAAAGCTTTTCC
 TGGCTCCGATCTCTGATTCCGACGAACTCTACAACACCATCCAGATCAAA
 GAATACGACGAACAGCCGACCTACTCTTGCCAGCTGCTGTTCAAGAAAG
 ATGAAGAATCTACTGACGAAATCGGTCTGATCGGTATCCACCGTTTCTAC
 GAATCTGGTATCGTATTCGAAGAATACAAGACTACTTCTGCATCTCCAA
 ATGGTACCTGAAGGAAGTTAAACGCAAACCGTACAACCTGAAACTGGGT
 TGCAATTGGCAGTTCATCCCGAAAGACGAAGGTTGGACCGAAtagtaaGAA
 TTC

B: BoNTB(H_c) encoded protein SEQ ID NO: 8

MANKYNSEILNNILNLRYKDNLDLSGYGAKVEVYDGVELNDKNQFKLT
 SSANSKIRVTQNQNIIFNSVFLDFSVSFWIRIPKYKNDGIQNYIHNEYTIINCM
 KNNSGWKISIRGNRIIWTLDINGKTKSVFFEYNIREDISEYINRWFFVTITNNL
 NNAKIYINGKLESNTDIKDIREVIANGEIIFKLDGDIDRTQFIWMKYFSIFNTEL
 SQSNIEERYKIQSYSEYLKDFWGNPLMYNKEYYMFNAGNKNSYIKLKKDSP
 VGEILTRSKYNQNSKYINYRDL YIGEFIRRKSNSSQSINDDIVRKEDIYILDF
 FNLNQEW RVYTYKYFKKEEEKLFLAPISDSDEL YNTIQIKEYDEQPTYSCQL
 LFKKDEESTDEIGLIGIHRFYESGIVFEEYKDYFCISKWYLKEVKRKPYNLKL
 GCNWQFIPKDEGWTE**

FIGURE 5

A: BoNTC₁(H_c) synthetic gene SEQ ID NO: 9

GAATTCACGatgACCATCCCATTCAACATCTTCTCCTACACCAACAACCTCC
 CTGTTGAAGGACATCATCAACGAGTACTTCAACAACATCAACGACTCCA
 AGATCCTGTCCCTGCAGAACCGTAAGAACACCTTGGTCGACACCTCCGG
 TTACAACGCCGAGGTCTCCGAGGAGGGTGACGTCCAGCTGAACCCAATC
 TTCCCATTCGACTTCAAGCTGGGTTCTCCGGTGAGGACAGAGGTAAGGT
 CATCGTCACCCAGAACGAGAACATCGTCTACAACTCCATGTACGAGTCC
 TTCTCCATCTCCTTCTGGATCAGAATCAACAAGTGGGTCTCCAACCTGCC
 AGGTTACACCATCATCGACTCCGTCAAGAACAACCTCCGGTTGGTCCATCG
 GTATCATCTCCAACCTTCTGGTCTTCAACCCTGAAGCAGAACGAGGACTCC
 GAGCAGTCCATCAACTTCTCCTACGACATCTCCAACAACGCTCCTGGTTA
 CAACAAGTGGTTCTTCGTCAACCGTCACCAACAACATGATGGGTAAACATG
 AAGATCTACATCAACGGTAAGCTGATCGACACCATCAAGGTCAAGGAGT
 TGACCGGTATCAACTTCTCCAAGACCATCACCTTCGAGATCAACAAGATC
 CCAGACACCGGTCTGATCACCTCCGACTCCGACAACATCAACATGTGGA
 TCCGTGACTTCTACATCTTCGCCAAGGAGTTGGACGGTAAGGACATCAA
 CATCCTGTTCAACTCCTTGCAGTACACCAACGTCGTCAAGGACTACTGGG
 GTAACGACCTGAGATACAACAAGGAGTACTACATGGTCAACATCGACTA
 CTTGAACAGATACATGTACGCCAACTCCAGACAGATCGTCTTCAACACC
 AGACGTAACAACAACGACTTCAACGAGGGTTACAAGATCATCATCAAGC
 GTATCAGAGGTAACACCAACGACACCAGAGTCAGAGGTGGTGACATCCT
 GTACTTCGACATGACTATCAACAACAAGGCCTACAACCTGTTTCATGAAG
 AACGAGACCATGTACGCCGACAACCACTCCACCGAGGACATCTACGCCA
 TCGGTCTGCGTGAGCAGACCAAGGACATCAACGACAACATCATCTTCCA
 GATCCAGCCAATGAACAACACTTACTACTACGCTTCCCAGATCTTCAAGT
 CCAACTTCAACGGTGAGAACATCTCCGGTATCTGTTCCATCGGTACCTAC
 AGATTCCGTCTGGGTGGTGACTGGTACAGACACAACCTACTTGGTTCCAAC
 TGTC AAGCAGGGTAACTACGCCTCCTTGCTGGAGTCCACTTCCACCCACT
 GGGGATTCTGTCCTCCAGTCTCCGAGtaaatagGAATTC

B: BoNTC₁(H_c) encoded protein SEQ ID NO: 10

MTIPFNIFS YTNNSLLKDIINEYFNNINDSKILSLQNRKNTLVDTSGYNAEVSE
 EGDVQLNPIFPDFKLGSSGEDRGKVIVTQENENIVYNSMYESFSISFWIRINK
 WWSNLPGYTIIDSVKNNSGWSIGIISNFLVFTLKQNEDEQSINFSDISNNAP
 GYNKWFFVTVTNMMGMNMIYINGKLIDTIKVKELTGINFSTITFEINKIPD
 TGLITSDSDNINMWIRDFYIFAKELDGKDINILFNSLQYTNVVKDYWGNDLR
 YNKEYYMVNIDYLNRYMYANSRQIVFNTRRNNNDFNEG YKIIKRIRGNTN
 DTRVRGGDILYFDMTINNKA YNLFMKNETMYADNHSTEDIYAIGLREQTKD
 INDNIIFIQPMNNTY YASQIFKSNFNGENISGICSIGTYRFRLLGGDWYRHN
 YLVPTVKQGN YASLLESTSTHWGFVPVSE**

FIGURE 6

A: BoNTD(H_c) synthetic gene SEQ ID NO: 11

GAATTCACGatgCGTTTGAAGGCTAAGGTCAACGAGTCCTTCGAGAACAC
CATGCCATTCAACATCTTCTCCTACACCAACAACCTCCTTGTTGAAGGACA
TCATCAACGAGTACTTCAACTCCATCAACGACTCCAAGATCTTGTCTTG
CAGAACAAGAAGAACGCCTTGGTCGACACCTCCGGTTACAACGCCGAGG
TCAGAGTCGGTGACAACGTCCAGTTGAACACCATCTACACCAACGACTT
CAAGTTGTCCTCTTCCGGTGACAAGATCATCGTCAACTTGAACAACAACA
TCTTGTAATCCGCCATCTACGAGAATCCTCTGTCTCCTTCTGGATCAAG
ATCTCCAAGGACTTGACCAACTCCCACAACGAGTACACCATCATCAACT
CCATCGAGCAGAACTCCGGTTGGAAGTTGTGTATCCGTAACGGTAACAT
CGAGTGGATCTTGCAGGACGTCAACCGTAAGTACAAGTCCTTGATCTTCG
ACTACTCCGAGTCCTTGTCCCACACCGGTTACACCAACAAGTGGTTCTTC
GTCACCATCACCAACAACATCATGGGTTACATGAAGTTGTACATCAACG
GTGAGTTGAAGCAGTCCCAGAAGATCGAGGACCTGGACGAGGTCAAGCT
GGACAAGACCATCGTCTTCGGTATCGACGAGAACATCGACGAGAACCAG
ATGTTGTGGATCCGTGACTTCAACATCTTCTCCAAGGAGCTGTCCAACGA
GGACATCAACATCGTCTACGAGGGTTCAGATCCTGAGGAACGTCATCAAG
GACTACTGGGGTAACCCACTGAAGTTCGACACCGAGTACTACATCATCA
ACGACAACATACATCGACCGTTACATCGCCCCAGAGTCCAACGTCCTGGT
CCTGGTCCAGTACCCTGACCGTTCCAAGCTGTACACCGGTAACCCTATCA
CCATCAAGTCCGTCTCCGACAAGAACCCTTACTCCCGTATCCTGAACGGT
GACAACATCATCCTGCACATGCTGTACAACTCCCGTAAGTACATGATCAT
CCGTGACACCGACACCATCTACGCCACCCAGGGTGGTGACTGTTCCCAG
AACTGTGTCTACGCCCTGAAGCTGCAGTCCAACCTGGGTAACCTACGGTAT
CGGTATCTTCTCCATCAAGAACATCGTCTCCAAGAACAAGTACTGCTCCC
AGATCTTCTCCTCCTTCCGTGAGAACACCATGCTGCTGGCCGACATCTAC
AAGCCTTGGCGTTTCTCCTTCAAGAACGCCTACACTCCTGTGCGCGTCAC
CAACTACGAGACCAAGCTGCTGTCCACCTCCTCCTTCTGGAAGTTCATCT
CCCGTGACCCAGGTTGGGTTCGAGtaaatagGAATTC

B: BoNTD(H_c) encoded protein SEQ ID NO: 12

MRLKAKVNESFENTMPFNIFS YTNNSLLKDIINEYFNSINDSKILSLQNKKNA
LVDTSGYNAEVRVGDNVQLNTIYTNDFKLSSSGDKIIVNLNNNIL YSAIYENS
SVSFWIKISKDLTNSHNEYTIINSIEQNSGWKLCIRNGNIEWILQDVNRKYKS
LIFDYSESLSHTGYTNKWFFVTITNNIMGYMKLYINGELKQSQKIEDLDEVK
LDKTIVFGIDENIDENQMLWIRDFNIFSKELSNEDINIVYEGQILRNVIKDYW
GNPLKFDTEYYIINDNYIDR YIAPESNVLVLVQYPDRSKLYTGNPITIKSVSD
KNPYSRILNGDNILHMLYNSRKYMIIRD TDTIYATQGGDCSQNCVYALKLQ
SNLGNYGIGIFSINKNIVSKNKYCSQIFSSFRENTMLLADIYKPWRFSFKNAYTP
VAVTNYETKLLSTSSFWKFISRDPGWVE**

FIGURE 7**A: BoNTE(H_c) synthetic gene SEQ ID NO: 13**

GAATTCACCatgGGAGAGAGTCAGCAAGAACTAAATTCTATGGTAACTGA
TACCCTAAATAATAGTATTCCTTTTAAGCTTTCTTCTTATACAGATGATAA
AATTTTAATTCCTACTTCAACAAGTTCTTCAAGAGAATTAAGTCTTCTTC
CGTTTTAAACATGAGATACAAGAATGATAAATACGTCGACACTTCCGGT
TACGACTCCAATATCAACATTAACGGTGACGTGTACAAGTACCCAACTA
ACAAAAACCAATTCGGTATCTACAACGACAAGCTTACTGAGCTGAACAT
CTCTCAAAACGACTACATTATCTACGACAACAAGTACAAGAACTTCTCTA
TTTCTTTCTGGGTCAGGATTCCTAACTACGACAACAAGATCGTCAACGTT
AACAAACGAGTACACTATCATCAACTGTATGAGAGACAACAACCTCCGGTT
GGAAGGTCTCTCTTAACCACAACGAGATCATTTGGACCTTGCAAGACAA
CGCAGGTATTAACCAAAAAGTTAGCATTCAACTACGGTAACGCAAACGGT
ATTTCTGACTACATCAACAAGTGGATTTTCGTCACTATCACTAACGACAG
ATTAGGTGACTCTAAGCTTTACATTAACGGTAACTTAATCGACCAAAAAGT
CCATTTTAAACTTAGGTAACATTCACGTTTCTGACAACATCTTATTCAAG
ATCGTTAACTGCAGTTACACCAGATACATTGGCATTAGATACTTCAACAT
TTTCGACAAGGAGTTAGACGAGACCGAGATTCAAACTTTATACAGCAAC
GAACCTAACACCAATATTTTGAAGGACTTCTGGGGTAACTACTTGCTTTA
CGACAAGGAATACTACTTATTAACGTGTAAAGCCAAACAACCTTCATT
GATAGGAGAAAGGATTCTACTTTAAGCATTAAACAACATCAGAAGCACTA
TTCTTTTAGCTAACAGATTATACTCTGGTATCAAGGTAAAGATCCAAAGA
GTAAACAACCTCTTCTACTAACGATAACCTTGTTAGAAAGAACGATCAGGT
CTATATTAACCTTCGTCGCTAGCAAGACTCACTTATTCCCATTATATGCTG
ATACCGCTACCACCAACAAGGAGAAGACCATCAAGATCTCCTCCTCTGG
CAACAGATTTAACCAAGTCGTCGTTATGAACTCCGTCGGTAACAACCTGTA
CCATGAACTTTAAAAATAATAATGGAAATAATATTGGGTTGTTAGGTTTC
AAGGCAGATACTGTAGTTGCTAGTACTTGGTATTATACCCACATGAGAG
ATCACACCAACAGCAATGGATGTTTTTGGAACTTTATTTCTGAAGAACAT
GGATGGCAAGAAAAA_{taa}
TagGGATCC GCGGCCGC ACGCGT CCCGGG ACTAGT GAATTC

B: BoNTE(H_c) encoded protein SEQ ID NO: 14

MGESQQELNSMVTDTLNNIPFKLSSYTDDKILISYFNKFFKRIKSSSVLNMR
YKNDKYVDTSGYDSNININGDVYKYPTNKNQFGIYNDKLTELNISQNDYIY
DNKYKNFSISFWVRIPNYDNKIVNVNNEYTIINCMRDNNSGWKVSLNHNEII
WTLQDNAGINQKLAFNYGNANGISDYINKWIFVTITNDRLGDSKLYINGNLI
DQKSILNLGNIHVSDNILFKIVNCSYTRYIGIRYFNIFDKELDETEIQTLYSNEP
NTNILKDFWGNLYLLYDKEYYLLNVLKPNNFIDRRKDSTLSINNIRSTILLANR
LYSGIKVKIQRVNNSSTNDNLVRKNDQVYINFAVASKTHLFPLYADTATTNKE
KTIKISSSGNRFNQVVMNSVGNNTMNFKNNGNNGNIGLLGFKADTVVAST
WYYTHMRDHTNSNGCFWNFISEEHGWQEK**

Ex. 1

AMINO ACID AND DESIGNED NUCLEOTIDE SEQUENCE OF BONTE(Hc)

AUG II
 TTC GAA ACG ATG ATT TTA ATT TCC TAC TTC AAC AAG TTC TTC AAG AGA ATT AAG TCT TCT TCC GTT TTA AAC ATG AGA TAC AAG AAT GAT AAA TAC GTC
 GAC ACT TCC GGT TAC GAC TCC AAT ATC AAC ATT AAC GGT GAC GTG TAC AAG TAC CCA ACT AAC AAA AAC CAA TTC GGT ATC TAC AAC GAC AAG CTT ACT GAG CTG
 D T S G Y D S N I N I N G D V Y K Y P T N K N Q F G I Y N D K L (1) E L
 AAC ATC TCT CAA AAC GAC TAC ATT ATC TAC GAC AAC AAG TAC TCT ATT TCT TTC TGG GTC AGA ATT CCT AAC TAC GAC AAC AAG ATC GTC AAC GTT
 N I S Q N D Y I I Y D N K Y K N F S I S F W V R I P N Y D N K I V N V
 AAC AAC GAG TAC ACT ATC ATC AAC TGT ATG AGA GAC AAC AAC TCC GGT TGG AAG GTC TCT CTT AAC CAC AAC GAG ATC ATT TGG ACC TTG CAA GAC AAC GCA GGT
 N N E Y T I I N C M R D N N S G W K V S L N H N E I I W T L Q D N A G
 ATT AAC CAA AAG TTA GCA TTC AAC TAC GGT AAC GCA AAC GGT ATT TCT GAC TAC ATC AAC AAG TGG ATT TTC GTC ACT ATC AAC GAC AGA TTA GGT GAC TCT
 I N Q K L A F N Y G N A N G I S D Y I N K W I F V T I T N D R L G D S
 AAG CTT TAC ATT AAC GGT AAC TTA ATC GAC CAA AAG TCC ATT TTA AAC TTA GGT AAC ATT CAC GTT TCT GAC AAC ATC TTA TTC AAG ATC GGT AAC TCC AGT TAC
 K L Y I N G N L I D Q K S I L N L G N I H V S D N I L F K I V N C S Y
 ACC AGA TAC ATT GGC ATT AGA TAC TTC AAC ATT TTC GAC AAG GAG TTA GAC GAG ACC GAG ATT CAA ACT TTA TAC ACC AAC GAA CCT AAC ACC AAT ATT TTG AAG
 T R Y I G I R Y F N I F D K E L D E T E I Q T L Y S N E P N T N I L K
 GAC TTC TGG GGT AAC TAC TTG CTT TAC GAC AAG GAA TAC TAC TTA AAC GTG TTA AAC CCA AAC AAC TTC ATT GAT AGG AGA AAG GAT TCT ACT TTA AGC ATT
 D F W G N Y L L Y D K E Y Y L L N V L K P N N F I D R R K D S T L S I
 AAC AAC ATC AGA ACG ACT ATT CTT TTA OCT AAC AGA TTA TAC TCT GGT ATC AAG GTT AAG ATC CAA AGA GTT AAC AAC TCT TCT ACT AAC GAT AAC CTT GTT AGA
 N N I R S T I L L A N R L Y S G I K V K I Q R V N N S S T N D N L V R
 AAG AAC GAT CAG GTC TAT ATT AAC TTC GTC OCT ACG AAG ACT CAC TTA TTA TAT OCT GAT ACC OCT AAC AAC GAG AAG ACC ATC AAG ATC TCC
 K N D Q V Y I N F V A S K T H L F P L Y A D T A T T N K E K T I K I S
 TCC TCT GGC AAC AGA TTT AAC CAA GTC GTC ATT ATG AAC TCC GGT AAC AAC TGT ACC ATG AAC TTT AAA AAT AAT AAT GGA AAT AAT ATT CCG TTG TTA GGT
 S S G N R F N Q V V M N S V G N N C T M N F K N N N G N N I G L L G
 TTC AAG GCA GAT ACT GTA GTT GCT AGT ACT TGG TAT TAT ACC CAC ATG AGA GAT CAC ACC AAC ACG AAT GGA TGT TTT TGG AAC TTT ATT TCT GAA GAA CAT GGA
 F K A D T V V A S T W Y Y T H M R D H T N S N G C F W N F I S E E H G
 TGG CAA GAA AAA TAA TAG GGA TCC
 W Q E K ter BamHI

Fig 8

FIGURE 9**A: BoNTF(H_c) synthetic gene SEQ ID NO: 15**

GAATTCACGatgTCCTACACCAACGACAAGATCCTGATCTTGTACTTCAAC
 AAGCTGTACAAGAAGATCAAGGACAACCTCCATCTTGGACATGAGATACG
 AAAACAATAAGTTCATCGACATCTCCGGTTACGGTCCAACATCTCCATC
 AACGGTGACGTCTACATCTACTCCACCAATAGAAACCAGTTCGGAATCT
 ACTCCTCCAAGCCTTCCGAGGTCAACATCGCTCAGAACAACGACATCAT
 CTACAACGGAAGATAACCAGAACTTCTCCATCTCCTTCTGGGTCCGTATCC
 CAAAGTACTTCAACAAGGTCAACCTGAATAACGAGTACACCATCATCGA
 CTGCATCCGTAAACAATAACTCCGGATGGAAGATCTCCCTGAACTACAAC
 AAGATCATCTGGACCCTGCAGGACACCGCCGGTAACAATCAGAAAGTTGG
 TCTTCAACTACACCCAGATGATCTCCATCTCCGACTACATCAACAAGTGG
 ATCTTCGTCACCATCACCAATAACCGTTTGGGAAACTCCAGAATCTACAT
 CAACGGTAACTTGATCGACGAGAAGTCCATCTCCAACCTGGGTGACATC
 CACGTCTCCGACAACATTTTGTTCAGATCGTCGGTTGTAACGACACCCG
 TTACGTCGGGATCCGTTACTTCAAAGTCTTCGACACTGAGTTGGGTAAGA
 CCGAGATCGAGACCTTGTACTCCGACGAGCCTGACCCATCCATCCTGAA
 GGACTTCTGGGGTAACTACCTGCTGTACAACAAACGTTACTACTTGCTGA
 ACTTGTGCGTACCGACAAGTCCATCACCCAGAACTCCAACCTTCTTGAAC
 ATCAACCAGCAGAGAGGTGTCTACCAGAAGCCAAACATCTTCTCCAACA
 CCAGATTGTACACCGGAGTCGAGGTCAATTATCAGAAAGAACGGATCTAC
 TGATATTTCCAACACCGATAACTTCGTCAGAAAGAACGATCTGGCTTACA
 TCAACGTTGTGACAGAGATGTGGAATACCGTCTGTACGCCGATATCTCT
 ATCGCCAAACCTGAAAAGATCATCAAGCTGATCCGTACCTCTAACTCTA
 ACAACTCTCTGGGACAAATCATCGTCATGGACTCCATCGGTAATAACTGT
 ACCATGAACTTCCAGAACAAACACGGTGGAAACATCGGTTTGTGGGTT
 TCCACTCCAACAACTTGGTCGCTTCCTCCTGGTACTACAACAACATCCGT
 AAGAACACCTCCTCCAACGGTTGCTTCTGGTCCTTCATCTCCAAGGAGCA
 CGGTTGGCAGGAGAACTaatagGAATTC

B: BoNTF(H_c) encoded protein SEQ ID NO: 16

MSYTNDKILILYFNKLYKKIKDNSILDMRYENNKFIDISGYGSNISINGDVYTY
 STNRNQFGIYSSKPSEVNIAQNNDIYNGRYQNFSISFWVRIPKYFNKVNLLN
 EYTIIDCIRNNNSGWKISLNYNKIIWTLQDTAGNNQKLVFNYSQMISISDYIN
 KWIFVTITNNRLGNSRIYINGNLIDEKISNLGDIHVSDNILFKIVGCNDTRYV
 GIRYFKVFDTELKTEIETLYSDEPDPSILKDFWGNLYLLYNKRYLLNLLRT
 DKSITQNSNFLNINQQRGVYQKPNIFSNTRLYTGVEVIIRKNGSTDISNTDNF
 VRKNDLAYINVVDRDVEYRLYADISIAKPEKIIKLIRTSNSNSNLGQIIVMSDI
 GNNCTMNFQNNNGGNIGLLGFHSNNLVASSWYNNIRKNTSSNGCFWSFIS
 KEHGWQEN**

FIGURE 10**A: BoNTG(H_c) synthetic gene SEQ ID NO: 17**

GAATTCACGatgAAGGACACCATCCTGATCCAGGTCTTCAACAACACTACATC
TCCAACATCTCCTCCAACGCCATCCTGTCCCTGTCCTACCGTGGTGGTTCG
TCTGATCGACTCCTCCGGTTACGGAGCCACCATGAACGTCGGTCCGACG
TCATCTTCAACGACATCGGTAACGGTCAGTTCAAGCTGAACAACCTCCGA
GAACTCCAACATCACCGCCCACCAGTCCAAGTTCGTCTGTCTACGACTCCA
TGTTGACAACCTTCTCCATCAACTTCTGGGTCCGTACCCCAAAGTACAAC
AACAAACGACATCCAGACCTACCTGCAGAACGAGTACACCATCATCTCCT
GTATCAAGAACGACTCCGGTTGGAAGGTCTCCATCAAGGGAAACCGTAT
CATCTGGACCCTGATCGACGTCAACGCCAAGTCCAAGTCCATCTTCTTCG
AGTACTCCATCAAGGACAACATCTCCGACTACATCAACAAGTGGTTCTCC
ATCACCATCACCAACGACCGTCTGGGTAAACGCCAACATCTACATCAACG
GTTCCCTGAAGAAGTCCGAGAAGATCCTGAACCTGGACCGTATCAACTC
CTCCAACGACATCGACTTCAAGCTGATCAACTGTACCGACACCACCAAG
TTCGTCTGGATCAAGGACTTCAACATCTTCGGTCGTGAGCTGAACGCCAC
CGAGGTCTCCTCCCTGTACTGGATCCAGTCCTCCACCAACACCCTGAAGG
ACTTCTGGGGAAACCCACTGCGTTACGACACCCAGTACTACCTGTTCAAC
CAGGGTATGCAGAACATCTACATCAAGTACTTCTCCAAGGCCTCCATGG
GTGAGACCGCCCCTCGTACCAACTTCAACAACGCCGCCATCAACTACCA
GAACCTGTACCTGGGTCTGCGTTTCATCATCAAGAAGGCCTCCAACCTCC
GTAACATCAACAACGACAACATCGTCCGTGAGGGTGACTACATCTACCT
GAACATCGACAACATCTCCGACGAGTCCTACCGTGTCTACGTCTGGTCA
ACTCCAAGGAGATCCAGACCCAGCTGTTCTCTGGCCCCAATCAACGACGA
CCCTACCTTCTACGACGTCCTGCAGATCAAGAAGTACTACGAGAAGACC
ACCTACAACTGTCAGATCCTGTGCGAGAAGGACACCAAGACCTTTCGGAC
TGTTCCGTATCGGTAAGTTCGTCAAGGACTACGGTTACGTCTGGGACACC
TACGACAACTACTTCTGTATCTCCCAGTGGTACCTGCGTCGTATCTCCGA
GAACATCAACAAGCTGCGTCTGGGATGTAAGTGGCAGTTCATCCCAGTC
GACGAGGGTTGGACCGAGtaaatagGAATTC

B: BoNTG(H_c) encoded protein SEQ ID NO: 18

MKDTILIQVFNNYISNISSNAILSLSYRGGRLIDSSGYGATMNVGSDVIFNDIG
NGQFKLNSENSENITAHQSKFVVYDSMFDNFSINFWVRTPKYNNNDIQTYL
QNEYTIISCIKNDSGWKVSIGNRHWTIDVNAKSKSIFFEYSIKDNISDYINK
WFSITITNDRLGANIIYINGSLKKSEKILNLDRLINSSNDIDFKLINCTDITTKFV
WIKDFNIFGRELNATEVSSLYWQSSNTNLKDFWGNPLRYDTQYYLNFQGM
QNIYIKYFSKASMGETAPRTNFNNAAINYQNLYLGLRFLIKKASNSRNINNDN
IVREGDYIYLNIDNISDESRYVYVLVNSKEIQTQLFLAPINDDPTFYDVLQIKK
YYEKTITYNQCILCEKDKTFGLFGIGKFVKDYGYVWDTYDNYFCISQWYLR
RISENINKLRLGCNWQFIPVDEGWTE**

FIGURE 11

A: BoNTA(H_N) synthetic gene SEQ ID NO: 19

atgGCTCTGAACGACCTGTGCATCAAAGTTAACAACCTGGGACCTGTTCTTC
 TCCCCGTCTGAAGACAACCTTCACTAACGACCTGAACAAAGGCGAAGAAA
 TCACCTCCGACACTAACATCGAAGCTGCTGAAGAAAACATCTCTCTGGA
 CCTGATCCAGCAGTACTACCTGACTTTCAACTTCGACAACGAACCGGAA
 AACATCTCCATCGAAAACCTGTCTTCCGACATCATCGGTCAGCTGGAAC
 GATGCCGAACATCGAACGCTTCCCGAACGGCAAGAAATACGAACTGGAC
 AAATACACCATGTTCCACTACCTGCGTGCTCAGGAATTCGAACACGGTA
 AATCTCGTATCGCTCTGACTAACTCCGTTAACGAAGCTCTGCTGAACCCG
 TCTCGCGTTTACACCTTCTTCTTCCGACTACGTTAAGAAAGTTAACAA
 AGCTACTGAAGCTGCTATGTTCCCTGGGTTGGGTTGAACAGCTGGTTTACG
 ACTTCACCGACGAAACTTCTGAAGTTTCCACCACTGACAAAATCGCTGAC
 ATCACTATCATCATCCCGTACATCGGCCCGGCTCTGAACATCGGTAACAT
 GCTGTACAAAGACGACTTCGTTGGTGCTCTGATCTTCTCTGGCGCTGTTA
 TCCTGCTGGAATTCATCCCGGAAATCGCTATCCCGGTTCTGGGTACCTTC
 GCTCTGGTTTCCTACATCGCTAACAAAGTTCTGACTGTTTCAGACCATCGA
 CAACGCTCTGTCTAAACGTAACGAAAAATGGGACGAAGTTTACAAATAC
 ATCGTTACTAACTGGCTGGCTAAAGTTAACTCAGATCGACCTGATCCG
 TAAGAAGATGAAAGAAGCTCTGGAAAACCAGGCTGAAGCTACTAAAGC
 TATCATCAACTACCAGTACAACCAGTACACCGAAGAAGAAAAGAACAAC
 ATCAACTTCAACATCGATGACCTGTCTCTAACTGAACGAATCCATCAA
 CAAAGCTATGATCAACATCAACAAATTCTGAACCAAGTGCTCTGTTTCT
 ACCTGATGAACTCTATGATCCCGTACGGCGTTAAACGCCTGGAAGACTTC
 GACGCTTCCCTGAAAGACGCTCTGCTGAAATACATCCGTGACAACCTACG
 GTACTCTGATCGGCCAGGTTGACCGTCTGAAAGACAAGGTTAACAACAC
 CCTGTCTACTGACATCCCGTTCCAGCTGTCCAAATACGTTGACAACCAGta
 a

B: BoNTA(H_N) encoded protein SEQ ID NO: 20

MALNDLCIKVNNWDLFFSPSEDNFTNDLNKGEEITSDTNIEAAEENISLDLIQ
 QYYLTFNFDNEPENISIENLSSDIIGQLELMPNIEFPNGKKYELDKYTMFHY
 LRAQEFEGHKSRIALTNSVNEALLNPSRVYTFSSDYVKKV NKATEAAMFL
 GWVEQLVYDFTDETSEVSTTDKIADITIIPYIGPALNIGNMLYKDDFVGALIF
 SGAVILLEFIPIAIPVLGTFALVSYANKVLT VQTIDNALS KRNEKWDEVYK
 YIVTNWLAKVNTQIDLIRKKMKEALENQAEATKAIINYQYNQYTEEEKNNI
 NFNIDDLSSKLNESINKAMININKFLNQCSVSYLMNSMIPYGVKRLED FDSL
 KDALLKYIRDNYGTLIGQVDR LDKVNNLTSTDIPFQLSKYVDNQ*

FIGURE 12

A: BoNTB(H₂) synthetic gene SEQ ID NO: 21

atgGCTCCAGGAATCTGTATCGACGTCGACAACGAGGACTTGTTCATC
GCTGACAAGAACTCCTTCTCCGACGACTTGTCCAAGAACGAGAGAATCG
AGTACAACACCCAGTCCAACCTACATCGAGAACGACTTCCCAATCAACGA
GTTGATCTTGGACACCGACTTGATCTCCAAGATCGAGTTGCCATCCGAGA
ACACCGAGTCCTTGACTGACTTCAACGTCGACGTCACGAGTCTACGAGAA
GCAACCAGCTATCAAGAAGATTTTCACCGACGAGAACACCATCTTCCAA
TACCTGTACTCTCAGACCTTCCCTTTGGACATCAGAGACATCTCCTTGAC
CTCTTCCTTCGACGACGCCCTGCTGTTCTCCAACAAGGTCTACTCCTTCTT
CTCCATGGACTACATCAAGACTGCTAACAAGGTCGTCGAGGCCGGTTTG
TTCGCTGGTTGGGTCAAGCAGATCGTCAACGATTTTCGTCATCGAGGCTAA
CAAGTCCAACACCATGGACAAGATTGCCGACATCTCCTTGATTGTCCCAT
ACATCGGTTTGGCCTTGAACGTCGGTAACGAGACCGCCAAGGGTAACTT
CGAGAACGCTTTCGAGATCGCTGGTGCCTCCATCTTGTTGGAGTTCATCC
CAGAGTTGTTGATCCAGTCGTCGGTGCCTTCTTGTTGGAGTCTACATC
GACAACAAGAACAAGATCATCAAGACCATCGACAACGCTTTGACCAAGA
GAAACGAGAAGTGGTCCGACATGTACGGTTTGATCGTCGCCCAATGGTT
GTCCACCGTCAACACCCAATTCTACACCATCAAGGAGGGTATGTACAAG
GCCTTGAACCTACCAGGCCCAAGCTTTGGAGGAGATCATCAAGTACAGAT
ACAACATCTACTCCGAGAAGGAGAAGTCCAACATTAACATCGACTTCAA
CGACATCAACTCCAAGCTGAACGAGGGTATTAACCAGGCCATCGACAAC
ATCAACAACCTTCATCAACGGTTGTTCCGTCTCCTACTTGATGAAGAAGAT
GATTCCATTGGCCGTCGAGAAGTTGTTGGACTTCGACAACACCCTGAAG
AAGAACTTGTTGAACCTACATCGACGAGAACAAGTTGTACTTGATCGGTT
CCGCTGAGTACGAGAAGTCCAAGGTCAACAAGTACTTGAAGACCATCAT
GCCATTCGACTTGTCCATCTACACCAACGACACCATCTTGATCGAGATGT
TCtaa

B: BoNTB(H₂) encoded protein SEQ ID NO: 22

MAPGICIDVDNEDLFFIADKNSFSDDLKNERIEYNTQSNYIENDFPINELILD
TDLISKIELPSENTESLTDFNVDPVYQPAIKKIFTDENTIFQYLYSQTFPLD
IRDISLTSSFDDALLFSNKVYSFFSMDYIKTANKVVEAGLFAGWVKQIVNDF
VIEANKSNTMDKIADISLIVPYIGLALNVGNETAAGNFENAFEIAGASILLEFI
PELLIPVVGAFLLSYIDNKNKIIKTIDNALTKRNEKWSMDMYGLIVAQWLST
VNTQFYTIKEGMYKALNYQAQALEEIKYRYNIYSEKEKSNINIDFNDINSL
NEGINQAIDNINNFINGCSVS YLMKKMIPLAVEKLLDFDNTLKKNLLNYIDE
NKL YLIGSAEYKSKVNKYLKTIMPFDL SIYTNDTILEMF*

FIGURE 13

A: BoNTC₁(H_N) synthetic gene SEQ ID NO: 23

atgTCCCTGTACAACAAGACCCTTGACTGTAGAGAGCTGCTGGTGAAGAA
 CACTGACCTGCCATTCATCGGTGACATCAGTGACGTGAAGACTGACATCT
 TCCTGCGTAAGGACATCAACGAGGAGACTGAGGTGATCTACTACCCAGA
 CAACGTGTCAAGTAGACCAAGTGATCCTCAGTAAGAACACCTCCGAGCAT
 GGACAACTAGACCTGCTCTACCCTAGTATCGACAGTGAGAGTGAGATCC
 TGCCAGGGGAGAATCAAGTCTTCTACGACAACCGTACCCAGAACGTGGA
 CTACCTGAACTCCTACTACTACCTAGAGTCTCAGAAGCTGAGTGACAAC
 GTGGAGGACTTCACTTTACGCGTTCAATCGAGGAGGCTCTGGACAACA
 GTGCAAAGGTGTACACTTACTTCCCTACCCTGGCTAACAAGGTGAATGCC
 GGTGTGCAAGGTGGTCTGTTCCCTGATGTGGGCAAACGACGTGGTTGAGG
 ACTTCACTACCAACATCCTGCGTAAGGACACACTGGACAAGATCTCAGA
 TGTGTCAAGCTATCATCCCCTACATCGGACCCGCACTGAACATCTCCAAT
 CTGTGCGTCGTGGAACTTCACTGAGGCATTTCGAGTCACTGGTGTCAAC
 ATCCTGCTGGAGGCATTCCCTGAGTTCACAATCCCTGCTCTGGGTGCATT
 CGTGATCTACAGTAAGGTCCAGGAGCGAAACGAGATCATCAAGACCATC
 GACAACTGTCTGGAGCAGAGGATCAAGAGATGGAAGGACTCCTACGAGT
 GGATGATGGGAACGTGGTTGTCCAGGATCATCACCCAGTTCAACAACAT
 CTCCTACCAGATGTACGACTCCCTGAACTACCAGGCAGGTGCAATCAAG
 GCTAAGATCGACCTGGAGTACAAGAAGTACTCCGGAAGCGACAAGGAG
 AACATCAAGAGCCAGGTTGAGAACCTGAAGAACAGTCTGGACGTCAAG
 ATCTCGGAGGCAATGAACAACATCAACAAGTTCATCCGAGAGTGCTCCG
 TCACCTACCTGTTCAAGAACATGCTGCCTAAGGTCATCGACGAGCTGAA
 CGAGTTCGACCGAAACACCAAGGCAAAGCTGATCAACCTGATCGACTCC
 CATAACATCATCCTGGTCGGTGAGGTCGACAAGCTGAAGGCAAAGGTAA
 ACAACAGCTTCCAGAACtaa

B: BoNTC₁(H_N) encoded protein SEQ ID NO: 24

MSLYNKTLDCRELLVKNTDLPFIGDISDVKTDIFLRKDINEETEVIIYYPDNVS
 VDQVILSKNTSEHGQLDLLYPSIDSESEILPGENQVFYDNRTQNVDYLNYY
 YLESQKLSDNVEDFTFTRSIEEALDNSAKVYTYFPTLANKVNAGVQGGLFL
 MWANDVVEDFTTNILRKDTLDKISDVSAIIPYIGPALNISNSVRRGNFTEAFA
 VTGVTILLEAFPEFTIPALGAFVIYSKVQERNEIITIDNCLEQRIKRWKDSYE
 WMMGTWLSRIITQFNNISYQMYDSLNYQAGAIKAKIDLEYKKYSGSDKENI
 KSQVENLKNSLDVKISEAMNNINKFIRECSVTYLFKNMLPKVIDELNEFDRN
 TKAKLINLIDSHNIIIVGEVDKLKAKVNNSFQN*

FIGURE 14**A: BoNTD(H₂) synthetic gene SEQ ID NO: 25**

atggccAACTCCCGTGACGACTCCACCTGCATCAAGGTCAAGAACAACAGA
 CTGCCATACGTTGCCGACAAGGACTCCATCTCCCAGGAGATCTTCGAGA
 ACAAGATCATCACCGACGAGACCAACGTTCAAACTACTCCGACAAGTT
 CTCTTTGGACGAGTCCATCCTGGACGGTCAGGTCCCAATCAACCCAGAG
 ATCGTCGACCCACTGTTGCCAAACGTCAACATGGAGCCATTGAACTTGCC
 AGGTGAGGAGATCGTCTTCTACGACGACATCACCAAGTACGTCGACTAC
 TTGAACTCCTACTACTACTTGGAGTCTCAAAAGTTGTCTAACAACGTCGA
 GAACATCACCTTGACCACCTCCGTCGAGGAGGCCTTGGGTACTCTAACA
 AGATCTACACCTTCCTGCCATCCTTGGCTGAGAAGGTTAACAAGGGTGT
 CAAGCTGGTTTGTTCCTGAACTGGGCCAACGAGGTCGTCGAGGACTTCA
 CCACCAACATCATGAAGAAGGACACCCTGGACAAGATCTCCGACGTCTC
 CGTCATCATCCCATACATCGGTCCAGCCTTGAACATCGGTAACCTCCGCCC
 TGAGAGGTAACCTCAACCAGGCCTTCGCCACCGCCGGTGTGCGCTTCCTG
 CTGGAGGGTTTCCCAGAGTTCACCATCCCAGCCCTGGGTGTCTTCACCTT
 CTA CTCTCCATCCAGGAGAGAGAGAAGATCATCAAGACCATCGAGAAC
 TGCTTGGAGCAGAGAGTCAAGAGATGGAAGGACTCCTACCAGTGGATGG
 TTTCCAACCTGGCTGTCCAGAATCACCAACCAATTCAACCACATCAACTAC
 CAGATGTACGACTCCCTGTCCTACCAGGCCGACGCCATCAAGGCCAAGA
 TCGACCTGGAGTACAAGAAGTACTCCGGTTCCGACAAGGAGAACATCAA
 GTCCAGGTCGAGAACCTGAAGAACTCCTTGGACGTCAAGATCTCCGAG
 GCCATGAACAACATCAACAAGTTCATCCGTGAGTGTTCGTCACCTACCT
 GTTCAAGAACATGCTGCCAAAGGTCATCGACGAGCTGAACAAGTTCGAC
 CTGAGAACCAAGACCGAGCTGATCAACCTGATCGACTCCCACAACATCA
 TCCTGGTTGGTGAGGTTGAC_{taa}

B: BoNTD(H₂) encoded protein SEQ ID NO: 26

MANSRDDSTCIKVKNNRLPYVADKDSISQEIFENKIITDETNVQNYSDKFSLD
 ESILDGQVPINPEIVDPLLPNVNMEPLNLPGEIIVFYDDITKYVDYLNYYYYL
 ESQKLSNNVENITLTTSVEEALGYSNKIYTFPLSLAEKVNKGVQAGLFLNWA
 NEVVEDFTTNIMKKDTLDKISDVSVIPIYGPALNIGNSALRGNFNQAFATAG
 VAFLEGFPEFTIPALGVFTFYSSIQEREKIIKTIENCLEQRVKRWKDSYQWM
 VSNWLSRITTQFNHINYQMYDSL SYQADAIKAKIDLEYKKYSGSDKENIKSQ
 VENLKNSLDVKISEAMNNINKFIRECSVTYLFKNMLPKVIDELNKFDLRTKT
 ELINLIDSHNILLVGEVD*

FIGURE 15**A: BoNTE(H_N) synthetic gene SEQ ID NO: 27**

atgTCCATCTGCATCGAGATCAACAACGGTGAGCTGTTCTTCGTGGCTTCC
GAGAACAGTTACAACGATGACAACATCAACACTCCTAAGGAGATTGACG
ACACCGTCACTTCTAACAACAACACTACGAAAACGACCTGGACCAGGTCAT
CCTAAACTTCAACTCCGAGTCCGCCCCCTGGTCTGTCCGACGAGAAGCTGA
ACCTGACCATCCAGAACGACGCTTACATCCCAAAGTACGACTCCAACGG
TACATCCGATATCGAGCAGCATGACGTTAACGAGCTTAACGTCTTCTTCT
ACTTAGACGCTCAGAAGGTGCCCGAGGGTGAGAACAACGTCAATCTCAC
CTCTTCAATTGACACAGCCTTGTTGGAGCAGCCTAAGATCTACACCTTCT
TCTCCTCCGAGTTCATCAACAACGTCAACAAGCCTGTGCAGGCCGCATTG
TTCGTAAGCTGGATTGAGCAGGTGTTAGTAGACTTCACTACTGAGGCTAA
CCAGAAGTCCACTGTTGACAAGATCGCTGACATCTCCATCGTCGTCCCAT
ACATCGGTCTGGCTCTGAACATCGGCAACGAGGCACAGAAGGGCAACTT
CAAGGATGCCCTTGAGTTGTTGGGTGCCGGTATTTTGTGAGTTCGAAC
CCGAGCTGCTGATCCCTACCATCCTGGTCTTACGATCAAGTCCTTCCTG
GGTTCCTCCGACAACAAGAACAAGGTCATTAAGGCCATCAACAACGCCC
TGAAGGAGCGTGACGAGAAGTGGAAGGAAGTCTATTCCTTCATCGTCTC
GAACTGGATGACCAAGATCAACACCCAGTTCAACAAGCGAAAGGAGCA
GATGTACCAGGCTCTGCAGAACAGGTCAACGCCATCAAGACCATCATC
GAGTCCAAGTACAACCTCTACACCCTGGAGGAGAAGAACGAGCTTACCA
ACAAGTACGATATCAAGCAGATCGAGAACGAGCTGAACCAGAAGGTCTC
CATCGCCATGAACAACATCGACAGGTTCTTGACCGAGTCCTCCATCTCCT
ACCTGATGAAGCTCATCAACGAGGTCAAGATCAACAAGCTGCGAGAGTA
CGACGAGAATGTCAAGACGTACCTGCTGAACTACATCATCCAGCACGGA
TCCATCCTGtaa

B: BoNTE(H_N) encoded protein SEQ ID NO: 28

MSICIEINNGELFFVASENSYNDDNINTPKEIDDTVTSNNNYENDLDQVILNF
NSESAPGLSDEKLNLTIQNDAYTPKYDSNGTSDIEQHDVNELNVFFYLDAQK
VPEGENNVLNTSSIDTALLEQPKIYTFSSSEFINNVNKPVQAALFVSWIQQVL
VDFTTEANQKSTVDKIADISIVVPYIGLALNIGNEAQKGNFKDALELLGAGIL
LEFEPELLIPTILVFTIKSFLGSSDNKNKVIKAINNALKERDEKWKEVYSFVS
NWMTKINTQFNKRKEQMYQALQNQVNAIKTIIESKYNSYTLLEKNELTNKY
DIKQIENELNQKVSAMNNIDRFLTESSISYLMKLINEVKINKLREYDENVKT
YLLNYIIQHGSIL*

FIGURE 16

A: BoNTF(H_N) synthetic gene SEQ ID NO: 29

atgGCCCCACCACGTCTGTGTATTAGAGTCAACAACCTCAGAATTATTCTTT
GTCGCTTCCGAGTCAAGCTACAACGAGAACGATATTAACACACCTAAAG
AGATTGACGATACTACCAACCTAAACAACACTACCGGAACAACCTGGA
TGAGGTTATTTTGGATTACAACCTCACAGACCATCCCTCAAATTTCCAACC
GTACCTTAAACACTCTTGTCCAAGACAACCTCCTACGTTCCAAGATACGAT
TCTAACGGTACCTCAGAGATCGAGGAGTATGATGTTGTTGACTTTAACGT
CTTTTTCTATTTGCATGCCCAGAAGGTGCCAGAAGGTGAAACCAACATCT
CATTGACTTCTTCCATTGATACCGCCTTGTGGAAGAGTCCAAGGATATC
TTCTTTTCTTCGGAGTTTATCGATACTATCAACAAGCCTGTCAACGCCGC
TCTGTTCAATTGATTGGATTAGCAAGGTCATCAGAGATTTTACCACTGAAG
CTACTCAAAAGTCCACTGTTGATAAGATTGCTGACATCTCTTGATTGTC
CCCTATGTCCGGTCTTGCTTTGAACATCATTATTGAGGCAGAAAAGGGTAA
CTTTGAGGAGGCTTTTGAATTGTTGGGAGTTGGTATTTTGTGAGTTTG
TTCCAGAACTTACCATTCTGTCAATTTTAGTTTTTACGATCAAGTCTTACA
TCGATTCATACGAGAACAAGAATAAAGCAATTAAGCTATTAACAACCTC
CTTGATCGAAAGAGAGGCTAAGTGGAAGGAAATCTACTCATGGATTGTA
TCAAACCTGGCTTACTAGAATTAACACTCAATTTAACAAGAGAAAGGAGC
AAATGTACCAGGCTCTGCAAAACCAAGTCGATGCTATCAAGACTGCAAT
TGAATACAAGTACAACAACCTATACTCCGATGAGAAGAACAGACTTGAA
TCTGAATACAATATCAACAACATTGAAGAAGAGTTGAACAAGAAAGTTT
CTTTGGCTATGAAGAATATCGAAAGATTTATGACCGAATCCTCTATCTCT
TACTTGATGAAGTTGATCAATGAGGCCAAGGTTGGTAAGTTGAAGAAGT
ACGATAACCACGTTAAGAGCGATCTGCTGAACTACATTCTCGACCACAG
ATCAATCCTGGGAGAGCAGACAAACGAGCTGAGTGATTTGGTTACTTCC
ACTTTGAACTCCTCCATTCCATTTGAGCTTTCTtaa

B: BoNTF(H_N) encoded protein SEQ ID NO: 30

MAPPRLCIRVNNSELFFVASESSYNENDINTPKEIDDTNLNNNYRNNLDEVI
LDYNSQTIPQISNRTLNTLVQDNSYVPRYDSNGTSEIEEYDVVDFNVFFYLH
AQKVPEGETNISLTSSIDTALLEESKDIFFSSEFIDTINKPVNAALFIDWISKVIR
DFTTEATQKSTVDKIADISLIVPYVGLALNIIIEAEKGNFEEAFELLGVGILLEF
VPELTIPVILVFTIKSYIDSYENKNKAIKAINNSLIEREAKWKEIYSWIVSNWL
TRINTQFNKRKEQMYQALQNQVDAIKTAIEYKYNNYTSDEKNRLESEYNIN
NIEEELNKKVSLAMKNIERFMTESISYLMKLINEAKVGKLKKYDNHVKSDL
LNYILDHRSILGEQTNELSDLVTSTLNSSIPFELS*

FIGURE 17

A: BoNTG(H_N) synthetic gene SEQ ID NO: 31

atggccAAAAATACCGGTAAATCTGAACAGTGTATTATTGTTAATAATGAG
 GATTTATTTTTCATAGCTAATAAAGATAGTTTTTCAAAAGATTTAGCTAA
 AGCAGAACTATAGCATATAATACACAAAATAACTATAGAAAATAAT
 TTTTCTATAGATCAGTTGATTTTAGATAATGATTTAAGCAGTGGCATAGA
 CTTACCAAATGAAAACACAGAACCATTACAAATTTTGACGACATAGAT
 ATCCCTGTGTATATTAACAATCTGCTTTAAAAAAATTTTTGTGGATGG
 AGATAGCCTTTTTGAATATTTACATGCTCAAACATTTCCCTTCTAATATAG
 AAAATCTACAATAACGAATTCATTAATGATGCTTTAAGAAATAATAA
 TAAAGTCTATACTTTTTTTTCTACAAACCTTGTTGAAAAAGCTAATACAG
 TTGTAGGTGCTTCACTTTTTGTAACTGGGTAAAAGGAGTAATAGATGAT
 TTTACATCTGAATCCACACAAAAAAGTACTATAGATAAAGTTTCAGATGT
 ATCCATAATTATTCCCTATATAGGACCTGCTTTGAATGTAGGAAATGAAA
 CAGCTAAAGAAAATTTTAAAAATGCTTTTGAAATAGGTGGAGCCGCTAT
 CTTAATGGAGTTTATTCCAGAACTTATTGTACCTATAGTTGGATTTTTTAC
 ATTAGAATCATATGTAGGAAATAAAGGGCATATTATTATGACGATATCC
 AATGCTTTTAAAGAAAAGGGATCAAAAATGGACAGATATGTATGGTTTGA
 TAGTATCGCAGTGGCTCTCAACGGTTAATACTCAATTTTATACAATAAAA
 GAAAGAATGTACAATGCTTTAAATAATCAATCACAAGCAATAGAAAAAA
 TAATAGAAGATCAATATAATAGATATAGTGAAGAAGATAAAATGAATAT
 TAACATTGATTTTAAATGATATAGATTTTAACTTAATCAAAGTATAAATT
 TAGCAATAAACAATATAGATGATTTTATAAACCAATGTTCTATATCATAT
 CTAATGAATAGAATGATTCCATTAGCTGTAAAAAAGTTAAAAGACTTTG
 ATGATAATCTTAAGAGAGATTTATTGGAGTATATAGATACAAATGAACT
 ATATTTACTTGATGAAGTAAATATTCTAAAATCAAAGTAAATAGACAC
 CTAAGACAGTATACCATTTGATCTTTCCTATATACCTaa

B: BoNTG(H_N) encoded protein SEQ ID NO: 32

MAKNTGKSEQCIIVNNEDLFFIANKDSFSKDLAKAETIAYNTQNNTIENNFSI
 DQLILDNDLSSGIDLPNENTEPFTNFDDIDIPVYIKQSALKKIFVDGDSLFEYL
 HAQTFPSNIENLQLTNSLNDALRNNNKVYTFSTNLVEKANTVVGASLFVN
 WVKGVIDDFTSESTQKSTIDKVSDVSIIPYIGPALNVGNETAKENFKNAFEIG
 GAAILMEFIPELIVPIVGFFTLESYVGNKGHIIMTISNALKKRDQKWTDMYGL
 IVSQWLSTVNTQFYTIKERMYNALNNQSQAIEKIIEDQYNRYSEEDKMNINI
 DFNDIDFKLNQSLNLAINNIDDFINQCSISYLMNRMIPLAVKKLKDFDDNLKR
 DLLEYIDTNELYLLDEVNILKSKVNRHLKDSIPFDLSLYT*

FIGURE 18**A: First BoNTF(Hc) gene: SEQ ID NO: 33**

GAATTCACGatgTCTTACACTAACGACAAAATCCTGATCCTGTACTTCAAC
AAACTGTACAAAAAATCAAAGACAACCTCTATCCTGGACATGCGTTACG
AAAACAACAAATTCATCGACATCTCTGGCTATGGTTCTAACATCTCTATC
AACGGTGACGTCTACATCTACTCTACTAACCAGCAACCAGTTCGGTATCTA
CTCTTCTAAACCGTCTGAAGTAAACATCGCTCAGAACAACGACATCATCT
ACAACGGTCGTTACCAGAACTTCTCTATCTCTTTCTGGGTTTCGTATCCCG
AAATACTTCAACAAAGTTAACCTGAACAACGAATACACTATCATCGACT
GCATCCGTAACAACAACCTCTGGTTGGAAAATCTCTCTGAACTACAACAA
AATCATCTGGACTCTGCAGGACACTGCTGGTAACAACCAGAACTGGTT
TTCAACTACACTCAGATGATCTCTATCTCTGACTACATTAATAAATGGAT
CTTCGTTACTATCACTAACAACCGTCTGGGTAACCTCTCGTATCTACATCA
ACGGTAACCTGATCGATGAAAAATCTATCTCTAACCTGGGTGACATCCA
CGTTTCTGACAACATCCTGTTCAAATCGTTGGTTGCAACGACACGCGTT
ACGTTGGTATCCGTTACTTCAAAGTTTTTCGACACTGAACTGGGTAAACT
GAAATCGAACTCTGTACTCTGACGAACCGGACCCGTCTATCCTGAAAG
ACTTCTGGGGTAACTACCTGCTGTACAACAACGTTACTACCTGCTGAAC
CTGCTCCGGACTGACAAATCTATCACTCAGAACTCTAACTTCCTGAACAT
CAACCAGCAGCGTGGTGTATATCAGAAACCTAATATCTTCTCTAACACTC
GTCTGTACACTGGTGTGAAGTTATCATCCGTAAAAACGGTTCTACTGAC
ATCTCTAACACTGACAACTTCGTACGTAAAAACGACCTGGCTTACATCAA
CGTTGTTGACCGTGACGTTGAATACCGTCTGTACGCTGACATCTCTATCG
CTAAACCGGAAAAAATCATCAAACCTGATCCGTACTTCTAACTCTAACAA
CTCTCTGGGTCAGATCATCGTTATGGACTCGATCGGTAACAACCTGCACTA
TGAACCTCCAGAACAAACAACGGTGGTAACATCGGTCTGCTGGGTTTCCA
CTCTAACAACTGGTTGCTTCTTCATGGTACTACAACAACATCCGTAAAA
ACACTTCTTCTAACGGTTGCTTCTGGTCTTTCATCTCTAAAGAACACGGTT
GGCAGGAAAACTaaGAATTC

B: BoNTF(Hc) #1 encoded protein: SEQ ID NO: 34

MSYTNDKILILYFNKLYKKIKDNSILDMRYENNKFIDISGYGSNISINGDVYY
STNRNQFGIYSSKPSEVNIAQNNDIYNGRYQNFSSISFWVRIPKYFNKVNLNN
EYTIIDCIRNNNSGWKISLNYNKIITLQDTAGNNQKLVFNQYTMISISDYIN
KWIFVTITNNRLGNSRIYINGNLIDEKSISNLGDIHVSDNLFKIVGCNDTRYV
GIRYFKVFDTELKTEIETLYSDEPDPSILKDFWGNLYLLYNKRYLLNLLRT
DKSITQNSNFLNINQQRGVYQKPNIFSNTRLYTGVEVIIRKNGSTDISTDNF
VRKNDLAYINVVDRDVEYRLYADISIAKPEKIIKLIRTSNSNNSLGQIIVMSI
GNNCTMNFQNNNGGNIGLLGFHSNNLVASSWYNNIRKNTSSNGCFWSFIS
KEHWQEN*

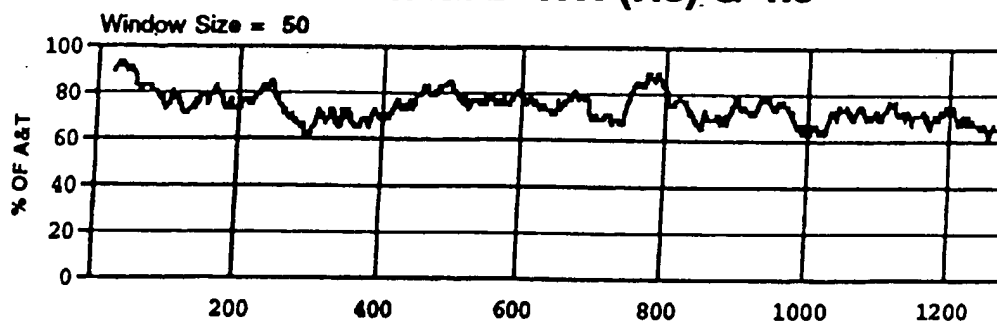
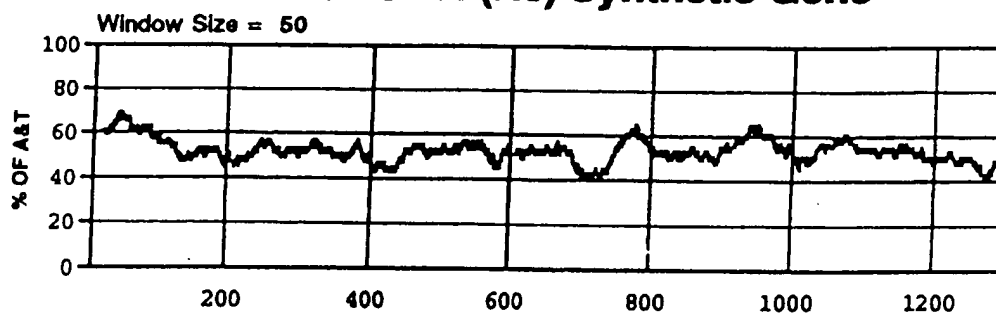
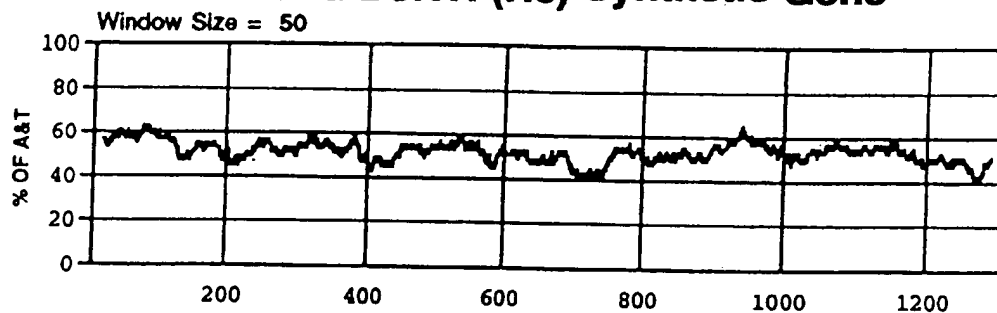
A: Natural B NTF(Hc) Gene**B: First BoNTF(Hc) Synthetic Gene****C: Second BoNTF(Hc) Synthetic Gene**

Fig 19

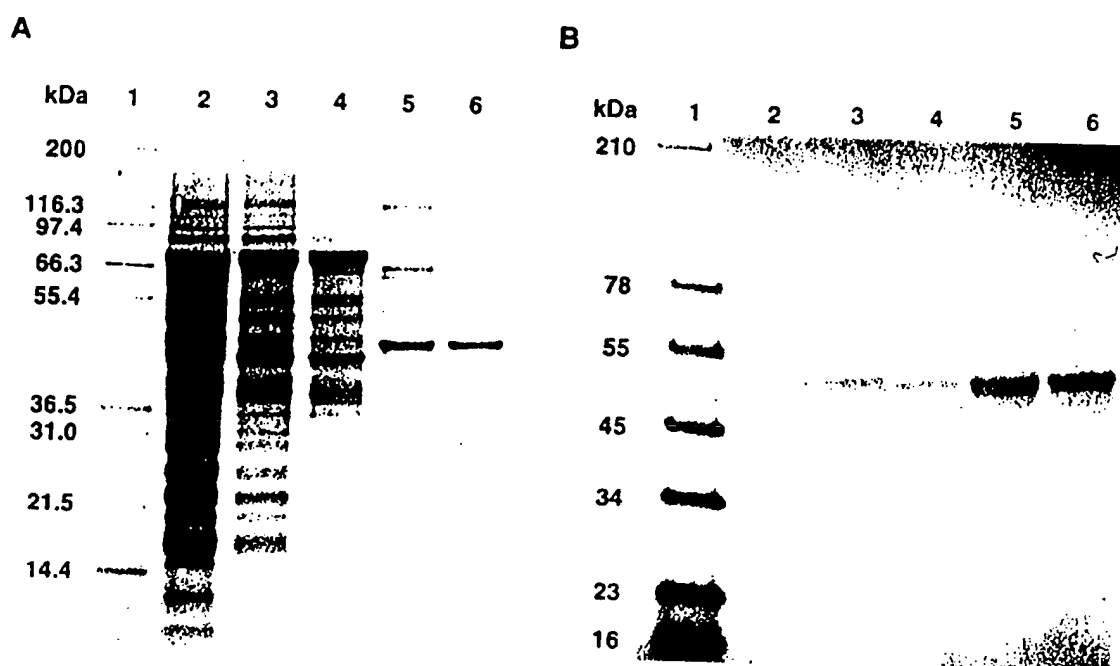


Fig 20

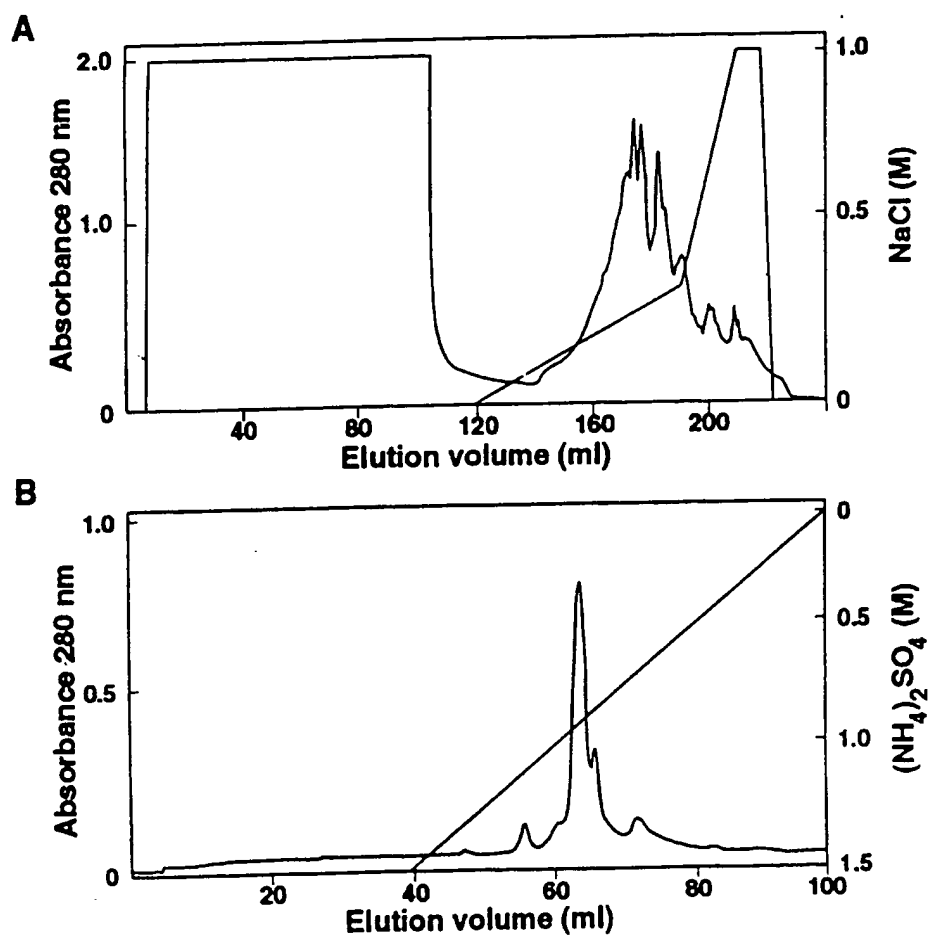


Fig 21

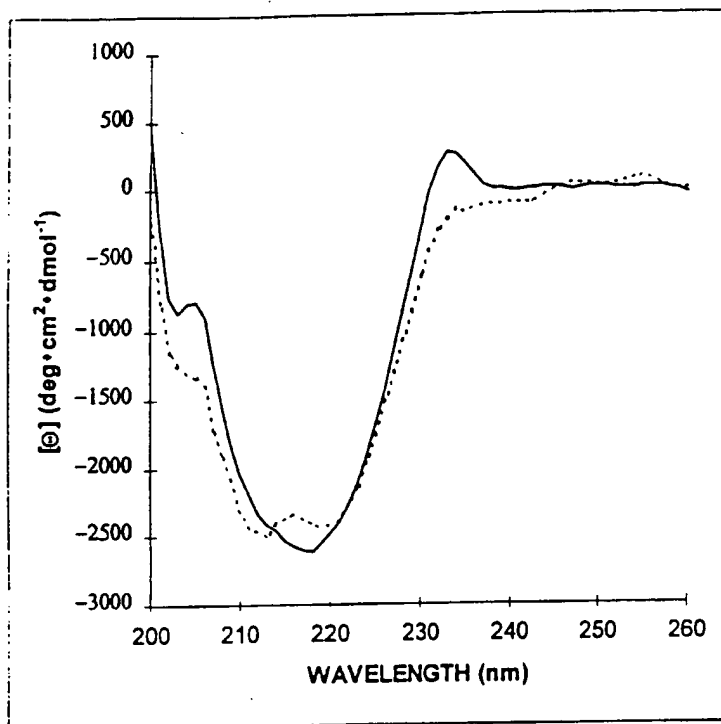


Fig 22

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
16 November 2000 (16.11.2000)

PCT

(10) International Publication Number
WO 00/67700 A3

- (51) International Patent Classification⁷: C12N 15/11, 15/63, C07H 21/04, C07K 5/00
- (21) International Application Number: PCT/US00/12890
- (22) International Filing Date: 12 May 2000 (12.05.2000)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:
- | | | |
|------------|---------------------------|----|
| 60/133,866 | 12 May 1999 (12.05.1999) | US |
| 60/133,873 | 12 May 1999 (12.05.1999) | US |
| 60/133,869 | 12 May 1999 (12.05.1999) | US |
| 60/133,865 | 12 May 1999 (12.05.1999) | US |
| 60/133,868 | 12 May 1999 (12.05.1999) | US |
| 60/133,867 | 12 May 1999 (12.05.1999) | US |
| 60/146,192 | 29 July 1999 (29.07.1999) | US |
- (71) Applicant (for all designated States except US): UNITED STATES ARMY MEDICAL RESEARCH & MATERIEL CMD [US/US]; 504 Scott Street, Fort Detrick, MD 21702-5012 (US).
- (72) Inventors; and
- (75) Inventors/Applicants (for US only): SMITH, Leonard, A. [US/US]; Clarksburg, MD (US). BYRNE, Michael, P. [US/US]; Frederick, MD (US). MIDDLEBROOK, John, L. [US/US]; Middletown, MD (US). LAPENOTIERE, Hugh [US/US]; Charlestown, WV (US).
- (74) Agents: POSORSKE, Laurence, H. et al.; Baker Botts, L.L.P., The Warner, 1299 Pennsylvania Avenue, N.W., Washington, DC 20004 (US).
- (81) Designated States (national): AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.
- (84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).
- Published:**
- With international search report.
 - Before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments.
- (88) Date of publication of the international search report:
8 February 2001
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*



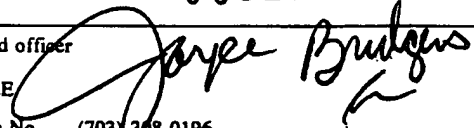
WO 00/67700 A3

(54) Title: RECOMBINANT VACCINE AGAINST BOTULINUM NEUROTOXIN

(57) Abstract: This invention is directed to preparation and expression of synthetic genes encoding polypeptides containing protective epitopes of botulinum neurotoxin (BoNT). The invention is also directed to production of immunogenic peptides encoded by the synthetic genes, as well as recovery and purification of the immunogenic peptides from recombinant organisms. The invention is also directed to methods of vaccination against botulism using the expressed peptides.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/12890

A. CLASSIFICATION OF SUBJECT MATTER IPC(7) : C12N 15/11, 15/63; C07H 21/04; C07K 5/00 US CL : 536/23.1, 23.4; 435/320.1, 252.3; 530/300 According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) U.S. : 536/23.1, 23.4; 435/320.1, 252.3; 530/300 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) WEST, STN, MEDLINE, BIOSIS, CAPLUS, botulinum neurotoxin, gene, type A, type B, DNA, sequence.		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LI, L. In Vitro Translation of Type A clostridium botulinum Neurotoxin Heavy Chain and Analysis of Its Binding to Rat Synaptosomes. J. Protein Chemistry. January 1999, Vol. 18, No. 1, pages 89-95, see entire document.	1, 4, 7-25
X	POTTER, K. et al. Production and Purification of the Heavy-Chain Fragment C of Botulinum Neurotoxin, Serotype B, Expressed in the Methylophilic Yeast Pichia pastoris. Protein Expression and Purification. 1998, Vol. 13, No. 3, pages 357-365, entire document.	1, 4, 7-25
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "B" earlier document published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search 31 OCTOBER 2000		Date of mailing of the international search report 06 DEC 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230		Authorized officer LI LEE  Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/12890

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CAMPBELL, K. et al. Gene Probes for Identification of the Botulinal Neurotoxin Gene and specific Identification of Neurotoxin Types B, E, and F. J. Clin. Micorbio. September 1993, vol. 31, No. 9, pages 2255-2262, entire document.	1, 4, 7-25

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/12890

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 2-3 and 5-6
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

No CRF for this case has been filed. Claims 2-3 and 5-6 which recite SEQ ID NOs cannot be searched other than by a sequence search.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.